BIRLA CENTRAL LIBRARY
PILANI | RAJASTHAN |

Class No. 612.0151

Book No. T182E

Accession No. 57011

This book has been
graciously presented by

Prof. M. L. Schroff

ENZYME CHEMISTRY

ENZYME CHEMISTRY

BY

HENRY TAUBER, Ph.D.
Consulting Chemist; formerly Instructor in Biochemistry,
New York Medical College and Flower Hospital

NEW YORK
JOHN WILEY & SONS, Inc.
London: CHAPMAN & HALL, Limited
1937

COPYRIGHT, 1937
BY
HENRY TAUBER

All Rights Reserved

This book or any part thereof must not be reproduced in any form without the written permission of the publisher.

PRINTED IN U S A

PRESS OF
BRAUNWORTH & CO INC.
BUILDERS OF BOOKS
BRIDGEPORT CONN.

PREFACE

During the last few years, the chemistry of enzymes has made rapid progress. The investigations of von Euler, Northrop, Sherman, Sumner, Warburg, Zeile, and their colleagues, and those of others, have proved that many enzymes are proteins. They are distinctly different from the heat-stable biochemical catalysts such as glutathione, adenylic acid, and ascorbic acid. The structure of compounds is often disclosed by means of enzyme action. Thus chlorophyll was crystallized and analyzed as a result of the action of the enzyme chlorophyllase. The chemistry of starch and proteins is becoming clearer, owing to more exact enzyme studies. Several enzymes and their precursors have been isolated in pure, crystalline form. This is an important step in enzymology. The discovery of a great number of oxidizing enzymes explains how inert (very stable) substances may be changed to readily oxidizable compounds *in vivo*. For these, specific coenzymes are often required. The study of the chemical composition of the latter has resulted in some interesting findings.

To preparative enzyme chemistry Willstätter and his collaborators made important contributions. They also improved and devised numerous methods for measuring enzyme activity.

The present book makes no claim to completeness. It is not possible to review all the work of even recent years, nor is it the author's intention to duplicate the material already presented in earlier monographs. It would seem to be more useful to present the more important of recent researches. *Theoretical considerations have been reduced to a minimum in this work.* For such the monograph by Haldane may be consulted.

The author acknowledges with gratitude the help he has received from Dr. Oscar Bodansky of New York University College of Medicine; Dr. F. A. Cajori of the University of Pennsylvania; Dr. O. M. Cope and Dr. I. S. Kleiner of New York Medical College and Flower Hospital; Dr. K. G. Falk of Bureau of Laboratories in the Health Department, New York; Dr. D. Glick of Mount Zion Hospital, San Francisco; Dr. J. M. Nelson and Dr. M. L. Caldwell of Columbia University; Dr. J. H. Northrop of The Rockefeller Institute for Medical Research; Dr. K. G. Stern of Yale University, who have been kind enough to show keen interest in reading and criticizing individual chapters.

Thanks are also due to the editors of the *American Journal of Diseases of Children*, the *Biochemical Journal*, *Die Naturwissenschaften*, the *Journal of the American Chemical Society*, the *Journal of Biological Chemistry*, the *Journal of General Physiology*, the *Journal of Nutrition*, the *Journal of Physiology*, and the *Zeitschrift für Physiologische Chemie*, who have granted their permission to reproduce figures and tables.

HENRY TAUBER

Long Island City, N. Y.

CONTENTS

CHAPTER PAGE

I. INTRODUCTION AND GENERAL CONSIDERATIONS
1. Definition of an enzyme........ 1
2. Effect of temperature........ 2
3. Effect of hydrogen ions; activity *p*H curves........ 4
4. Specificity of enzymes........ 6
5. Law of mass action........ 7
6. Monomolecular reaction........ 8
7. Effect of enzyme concentration on reaction velocity........ 9
8. Equation of Northrop........ 9
9. Michaelis-Menten theory; activity *pS* curves........ 10
10. Kinetics of hydrolysis of crude and crystalline trypsin........ 12
11. General rule for expressing enzyme activity........ 12
12. Activators and inhibitors........ 12
13. Antiseptics........ 13
14. Kinetics of inhibition........ 13
15. Inhibition number........ 14
16. Reversible inactivation........ 15
17. Chemical nature of enzymes........ 17
18. The carrier theory........ 19
19. Digestion and inactivation of enzymes by proteases........ 20
20. Views concerning the mechanism of enzyme action........ 21
21. Synthesis by enzymes........ 22
22. Preparation of enzyme material........ 23

II. ESTERASES
1. Pancreatic lipase........ 29
2. Pancreatic prolipase........ 30
3. The stereospecificity of esterases........ 30
4. Gastric lipase........ 32
5. Liver lipase (esterase)........ 33
6. Experiments showing differences between the liver and pancreatic enzyme........ 33
7. Ricinus lipase........ 35
8. Chlorophyllase........ 35
9. Tannase........ 36
10. Sulfatase........ 36
11. Phosphatases........ 37
12. Phosphatases in diseases........ 38
13. Choline esterase........ 39
14. Sym's method for the enzymic synthesis of esters........ 42

CHAPTER PAGE
III. PROTEOLYTIC ENZYMES AND PEPTIDASES
(A) 1. The structure of the protein molecule and its relation to proteolysis 49
2. Facts in support of a polypeptide structure 49
3. Facts in support of a diketopiperazin structure 50
4. Theory of Shibata 51
(B) 5. Mammal gastric proteases 53
6. The rennin-pepsin problem. Propepsin or pepsinogen and prorennin 53
7. Preparation of crystalline pepsinogen 54
8. Chemical differences between rennin and pepsin 55
9. Gastric proteases are kind specific 56
10. Preparation of specific gastric chymoinhibitors 56
11. The chemistry of milk clotting 58
12. Estimation of rennet activity 59
13. Crystalline pepsin 60
14. Preparation of crystalline pepsin 60
15. Chemical nature of crystalline pepsin 61
16. Crystalline acetyl derivatives of pepsin 63
17. Optimum *p*H of pepsin with various substrates 64
(C) 18. Tryptases 66
19. Enterokinase 67
20. Desmo-trypsin and lyo-trypsin 68
21. Crystalline trypsin 69
22. The effect of the substrate concentration on tryptic hydrolysis 70
23. Crystalline chymotrypsinogen and crystalline chymotrypsin 71
24. Preparation of crystalline chymotrypsinogen 72
25. Activation of chymotrypsinogen 73
26. Isolation and crystallization of chymotrypsin 74
27. Crystalline trypsinogen 76
28. Conversion of trypsinogen to trypsin and crystallization of trypsin 77
29. Crystalline trypsin inhibitor and crystalline inhibitor-trypsin compound 81
30. Autolytic trypsin 81
31. Papainases (kathepsin and papain) 84
32. The specificity of papain 86
33. The papain-activating power of blood in cancer 87
(D) 34. Peptidases 88
35. Polypeptidases 88
36. Aminopolypeptidase 88
37. Pancreatic polypeptidase 90
38. Prolinase or prolylpeptidase 91
39. Carboxypolypeptidase 92

CHAPTER PAGE

III. PROTEOLYTIC ENZYMES AND PEPTIDASES—(*Continued*)

(*D*) 40. Crystalline carboxypolypeptidase 92
41. Dipeptidase 93
42. Dehydropeptidase 94
43. Bromelin, keratinase and other proteolytic enzymes 94
44. Classification of proteolytic enzymes and peptidases 95

IV. AMIDASES

1. Asparaginase 106
2. Aspartase 107
3. Tyraminase 107
4. Urease 107
5. The intermediate product of urease action 108
6. Specificity of urease 109
7. Preparation of crystalline urease 109
8. The chemical nature of crystalline urease 110
9. Activators and inhibitors of urease 111
10. Reaction course of urease 113
11. Physiological properties of urease 113
12. The effect of buffers upon urease action 114
13. Hippuricase or histozyme 114
14. Arginase 115
15. Source of arginase 115
16. Preparation of crude arginase 115
17. Preparation of purified arginase 116
18. Activation and estimation of arginase 116
19. Mechanism of arginase action 116
20. Purine amidases 118

V. CARBOHYDRASES

(*A*) Sucrase (saccharase, invertase) 125
1. Preparation of sucrase 125
2. Methods for the estimation of sucrase activity 127
3. The specificity of hexosidases 128

(*B*) α-*d*-Glucosidases: maltases 129
1. Purification of yeast maltase and its separation from sucrase 130
2. Synthetic action of maltase 130
3. Estimation of maltase activity 130

(*C*) β-*d*-Galactosidases: lactase, melibiase 131
1. Lactase 131
2. Optimum activity 132
3. Reaction course of lactase 132
4. Effect of substrate concentration 132
5. Estimation of activity 134
6. Melibiase 134

CHAPTER PAGE

V. CARBOHYDRASES—(*Continued*)

(*D*) "Emulsin" 134
  1. The specificity of emulsin 134
  2. Effect of neutral salts upon emulsin 136
  3. Preparation of emulsin 136

(*E*) Polyases 137
  I. Amylases 137
    1. Kuhn's amylase specificity theory 138
    2. The two-starch component theory of Van Klinkenberg 138
    3. Lyo- and desmo-amylases of Willstätter and Rohdewald 139
    4. Influence of salts upon the optimum *p*H of amylase. Other activators 139
    5. Kinetics of starch hydrolysis 142
    6. Estimation of amylolytic activity 142
    7. Purification of pancreatic amylase and its chemical nature 143
    8. Crystalline pancreatic amylase 144
    9. Separation of α- and β-malt amylase and their purification 144
  II. Lichenase, cellulase, inulase 146

VI. CATALASE

1. Preparation of catalase 157
2. Activity as influenced by the enzyme and substrate concentration 158
3. Optimum *p*H of catalase 159
4. Chemical nature of catalase 159
5. Evidence for the formation of an enzyme-substrate compound 162
6. Activity *pS* curve for catalase 164

VII. OXIDIZING ENZYMES

(*A*) Dehydrogenases or anaerobic oxidases

  1. Wieland's theory 167
  2. Warburg's theory 168
  3. Succinic dehydrogenase 169
  4. Malic dehydrogenase 170
  5. Lactic dehydrogenase 170
  6. β-Hydroxybutyric dehydrogenase 171
  7. Citric dehydrogenase 172
  8. Alcohol dehydrogenase 172
  9. Glycerophosphoric dehydrogenase 173
  10. Hexosediphosphoric dehydrogenase 173
  11. Schardinger aldehyde dehydrogenase and xanthine dehydrogenase 174
  12. Glucose dehydrogenase 175

CHAPTER PAGE

VII. OXIDIZING ENZYMES—(*Continued*)
13. Amino acid dehydrogenase........................ 176
14. The methylene blue technic of Thunberg and Ahlgren 176

(*B*) Oxidases or aerobic oxidases
1. Peroxidases. Their importance as biological oxidants 178
2. Color reactions.................................. 179
3. Interaction of ascorbic acid and peroxidase......... 180
4. Preparation of peroxidase......................... 182
5. Estimation of peroxidase.......................... 183
6. Effect of *p*H on peroxidase....................... 183
7. Chemical nature of peroxidase..................... 183
8. Tyrosinase or monophenol oxidase................. 184
9. Optimum *p*H of tyrosinase........................ 185
10. The effect on phenols............................ 185
11. Dopa oxidase.................................... 185
12. Laccase or polyphenol oxidase.................... 186
13. Indophenol oxidase.............................. 187
14. Uricase... 187
15. Ascorbic acid (vitamin C) oxidase................ 188
16. Optimum *p*H of ascorbic acid oxidase............. 189
17. Determination of ascorbic acid oxidase............ 189
18. Interaction of glutathione in the enzymic oxidation of ascorbic acid.................................... 189
19. Other vitamins affecting enzymes.................. 190
20. Some of the functions of cytochrome in relation to oxidation...................................... 193

VIII. THE FLAVIN OXIDATION SYSTEM OF WARBURG AND CHRISTIAN AND ITS RELATION TO OTHER DYES
1. Preparation of the components and their properties......... 203
2. Crystallization of the yellow ferment....................... 205
3. Reversible hydrolysis of the enzyme....................... 206
4. Formation of the active chromoprotein from the synthetic prosthetic group and the specific protein................ 206

IX. THE ZYMASE COMPLEX AND ALCOHOLIC FERMENTATION
1. Zymase is a complex of enzymes and coenzymes............ 211
2. Estimation of activity.................................. 212
3. Induction... 213
4. Cozymase... 213
5. Isolation of cozymase.................................. 214
6. The rôle of inorganic phosphate in alcoholic fermentation... 215
7. Intermediary products of fermentation.................... 216

X. CARBONIC ANHYDRASE
1. Preparation of the enzyme............................... 223
2. Preparation of purified enzyme.......................... 224

CHAPTER PAGE

X. CARBONIC ANHYDRASE—(*Continued*)

3. Method for estimation of activity........................ 224
4. The enzyme is specific.................................. 226
5. Chemical nature... 226

XI. LUCIFERASE

1. Luciferin.. 227
2. Preparation of luciferase................................ 227
3. Mechanism of luciferase action......................... 228
4. Chemical nature of luciferin............................ 228
5. Chemical nature of luciferase........................... 228
6. Methods for estimation of luciferase activity.............. 229

AUTHOR INDEX... 231

SUBJECT INDEX.. 239

ENZYME CHEMISTRY

CHAPTER I

INTRODUCTION AND GENERAL CONSIDERATIONS

Enzymes are indispensable constituents of all living cells. Bechhold (1)* pictures the living cell as " a city in which colloids are the houses and the crystalloids are the people who traverse the streets, disappearing into and emerging from the houses, or who are engaged in demolishing or erecting buildings. The colloids are the stable parts of the organism, the crystalloids the mobile part, which penetrating everywhere may bring weal or woe." It is in this activity that the enzymes play an important part. The enzymes themselves, however, are cell independent. They have often been called biochemical catalysts. There are many biochemical catalysts. To assure a distinct differentiation between enzymic and nonenzymic catalysts, the author proposed the following classification (2):

Definition of an Enzyme. Classification of Biochemical Catalysts

1. *Specific, cell-independent, biochemical catalysts or enzymes:* Catalysts which are produced by the living cell, but whose action is independent of the living cell, and which are destroyed if their solutions are heated long enough. Examples: pepsin, trypsin, maltase, lipase.

2. *Specific, cell-dependent, biochemical catalysts:* Cata-

*** Numbers in parentheses refer to items in References at end of chapters.**

lysts produced by the living cell, active *in vitro* as well as *in vivo*, their activity, however, depending on the unimpaired cell. They are destroyed on heating, and their activity ceases on mechanical destruction of the cell. Example: the catalyst affecting the synthesis of urea in the liver (3), and the dehydrogenetic function of certain bacteria, which is inactivated on cell destruction (toluol)(4).

3. *Non-specific biochemical catalysts:* Catalysts elaborated by the living cell, their action being independent of the life process of the cell. They are not destroyed when their solutions are heated. Examples: glutathione, ascorbic acid. Von Szent-Györgyi (5) has recently suggested cytochrome and adenylic acid as classical examples of non-enzymic catalysts of the cell.

Effect of Temperature

The velocity of enzyme action is accelerated as the temperature is increased until an optimum is attained above which there is a decrease in velocity and enzyme activity discontinues. According to Arrhenius (6), velocity changes effected by temperature are due to two kinds of molecules in the solution, i.e., active and inactive ones, which are in tautomeric equilibrium. This view of activated molecules has been recently extended by the application of the quantum theory. The latter identifies the tautomeric activated forms with higher quantum states of the molecules. With each 10° increase in temperature there is an increase in enzymic activity of about two to three times the original. The relation between reactions at two temperatures 10° apart is called the temperature coefficient. The estimation should be carried out at temperatures where destruction is at a minimum.

In the following a typical experiment on the effect of temperature on the rate of reaction is given (6*a*). The enzyme employed was catalase, and the substrate was monoethyl hydrogen peroxide (a substituted peroxide).

Excess substrate was determined iodometrically and the amount of substrate decomposed is expressed in cubic centimeters of 0.1 *N* thiosulfate. A water thermostate was employed which could be adjusted to any desired temperature between +0.1° and +99° with a constancy of ±0.003°. The reaction time was five minutes in all experiments. The value for 0 time was obtained by adding sulfuric acid to the enzyme before the substrate was added. Four temperatures were selected and the tests were performed in quadruplicates, the average obtained of the four tests being used for the plotting of Fig. 1. From Fig. 1 it can be seen that the relationship between temperature and the rate of reaction (decomposition of the substrate) is linear in this case. Q_{10} in the interval 0–10° equals 2.3 and between 10–20° it is 2.19.

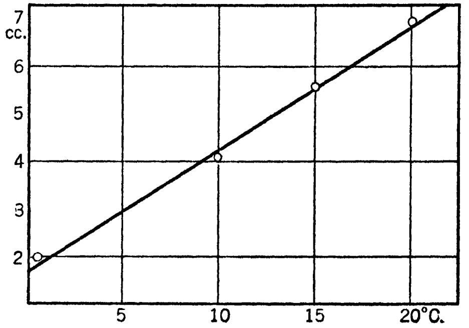


FIG. 1.—Variation of the reaction velocity with the temperature. The ordinate represents the amounts of substrate decomposed in 5 minutes, expressed in cc. of 0.1 *N* thiosulfate.

In Table I are presented a number of dependable temperature coefficients (K_{t+10}/K_t; or Q_{10}). The temperature coefficient for enzymic reactions is less than when the same reactions are catalyzed by hydrogen ions. Above 50° enzymes in solution are rapidly inactivated. The destruction increases with an increase in temperature, and with most enzymes it is complete at 80°. Some enzymes are more resistant, for instance, papain, bromelin (11), and the rennin of the plant *Solanum elaeagnifolium* (12). The concentration of the enzyme as well as the substrate and the duration of the experiment, however, are influential factors, and in some cases under certain conditions heat inactivation is reversible (e.g., trypsin). Dry enzyme preparations can stand temperatures of 100° to 120°.

TABLE I

TEMPERATURE COEFFICIENTS OF A NUMBER OF ENZYME ACTIONS

Enzyme	Substrate	Temperature in degrees	$\frac{K_{t+10}}{K_t}$	Reference number
Pancreatic lipase...	Ethyl butyrate	0–10	1.50	(7)
		10–20	1.34	
		20–30	1.26	
Liver lipase........	Ethyl butyrate	0–10	1.72	
		10–20	1.36	
		20–30	1.10	
Yeast maltase......	Maltose	10–20	1.90	(8)
		20–30	1.44	
		30–40	1.28	
Malt amylase......	Starch	20–30	1.96	(9)
		30–40	1.65	
Succinic oxidase....	Succinate	30–40	2.0	(10)
		40–50	2.1	
		50–60	2.1	

Effect of Hydrogen Ions; Activity *p*H Curves

Activity *p*H curves represent the influence of hydrogen-ion concentration upon the relative enzyme activity. The *p*H optima, however, will change according to the condition of the experiment. This point is clearly illustrated in Fig. 2; urease is most active in the presence of 1 per cent urea and $M/8$ citrate buffer at *p*H 6.5. The *p*H optimum for urease in the presence of 2.5 per cent urea is 6.4 for acetate, 6.5 for citrate, and 6.9 for phosphate. Urease is active from *p*H 5 to 9 in phosphate buffer, from 4 to 8.5 in citrate buffer, and 3 to 7.5 in acetate buffer [Howell and Sumner (13)].

In Fig. 3 the optimum *p*H of *two amylases of barley malt* is shown as determined by their saccharogenic (maltose-

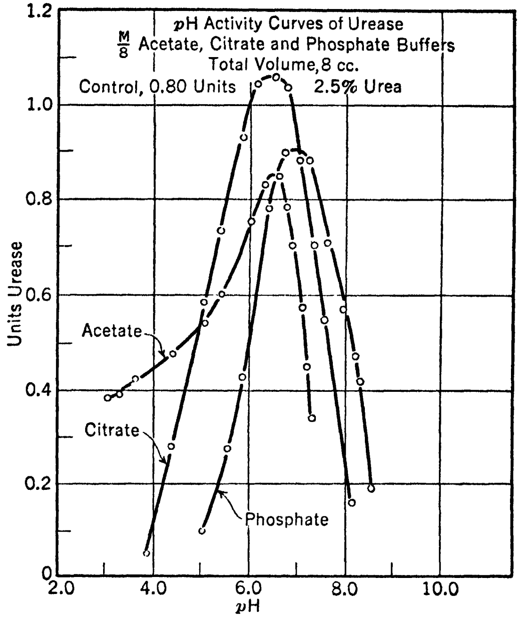


FIG. 2

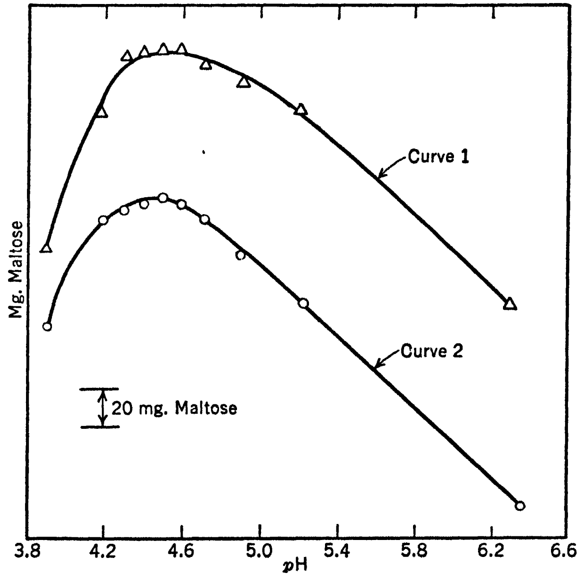


FIG. 3.—Optimum pH of two amylases of barley malt

forming) activity from 2 per cent starch in the presence of 0.01 *M* acetate at 40° in 30 minutes. Curve 1 represents a malt amylase preparation with preponderance of high initial saccharogenic activity; Curve 2 represents a malt amylase preparation with preponderance of high initial amyloclastic activity (decomposition of that part of the starch molecule which forms colored complexes with iodine). Under these conditions the optimum *p*H of saccharogenic action was 4.3 to 4.6, whereas the optimum of amyloclastic action under similar conditions was at 4.3 to 4.7 (14).

Table II shows several examples in which the optimum *p*H of an enzyme varies with the substrate, the source of enzyme material, and the buffer employed. Since these factors were not taken into consideration in many earlier studies a comprehensive tabulation of *p*H optima was not attempted.

Specificity of Enzymes

Enzymes differ from inorganic catalysts in that the latter catalyze many reactions; i.e., H ions (of acids) will hydrolyze proteins, fats, and carbohydrates, and any ester. OH ions behave similarly. Colloidal platinum, too, catalyzes many kinds of reactions. Enzymes, however, are more specific.

Lipases do not split proteins, and proteolytic enzymes do not attack fats. Thus their action is limited to certain types of substances. Some enzymes are absolutely specific. For instance, dipeptidase will not split a dipeptide if the NH_2 or COOH group is not free (Grassmann and Dyckerhoff). Some enzymes show stereospecificity; e.g., the natural form of certain compounds is attacked much faster than the synthetic antipode. Other enzymes prefer to open certain linkages; i.e., α- and β-amylase (Kuhn). There are two types of maltases: true α-glucosidases and pseudo α-glucosidases, each with specific properties (Kleiner and Tauber). There are enzymes, however, which can act on a great variety of compounds (e.g., emulsin), and Helferich

TABLE II

Showing That the Optimum *p*H Varies with the Type of Substrate, the Buffer, and the Enzyme Material (Source)

Enzyme	Optimum *p*H	Authority *
Amylase, pancreatic	6.8	Sherman, Thomas, and Baldwin
malt	4.4–5.2	Sherman and Schlesinger
salivary, acetate buffer	5.6	Hahn and Michaelis
phosphate buffer	6.5	Hahn and Meyer
Ascorbic acid oxidase, squash,		
phosphate-citrate buffer	5.56–5.93	Tauber, Kleiner, and Mishkind
acetate buffer	5.38–5.57	Tauber, Kleiner, and Mishkind
Lactase, adult dog intestine	5.4–6.0	Cajori
calf intestine	5.0	Fendenberg and Hoffman
cockroach, gut	5.0–6.4	Wigglesworth
yeast	7.0	Willstätter and Oppenheimer
almond	4.2	Willstätter and Csanyi
Maltase, yeast	6.75–7.25	Willstätter and Bamann
Solanum indicum	5.5	Tauber and Kleiner
Pepsin, egg albumin as substrate	1.5	Sørensen
casein as substrate	1.8	Northrop
hemoglobin as substrate	2.2	Northrop
gelatin as substrate	2.2	Northrop
Phosphatase, bone	8.4	Martland and Robinson
kidney	8.8–9.2	Kay
plant sources	3.4–6.0	Kay and Lee
synthetic action	9.4	Kay
Sucrase, yeast	4.5	Michaelis and Davidsohn
Solanum indicum	6.0	Tauber and Kleiner
Urease, crystalline; citrate buffer	6.5	Howell and Sumner
acetate buffer	6.4	Howell and Sumner
phosphate buffer	6.9	Howell and Sumner

* For further data and references see individual chapters.

compares such enzymes to the master key opening many locks.

Law of Mass Action

Enzymic reactions are influenced by many factors, the more complicated of which are not understood, for example, the nature of reacting or active groups in the enzyme molecule, the possibility of more than one kind of active group in the enzyme molecule, the quality and quantity of accompanying substances, which may be either activators or inhibitors. These are the factors which make an enzymic reaction differ quantitatively but not qualitatively from the inorganic catalyst. The application of the mass-action law, therefore, is limited in enzyme studies.

According to the mass-action law the reaction velocity is proportional to the concentration of the reacting molecules at any given time. The rate of change of one molecule at any time should be proportional to the unchanged substance.

Monomolecular Reaction. A reaction in which one substance is undergoing change is called monomolecular. It may be expressed by the following equation:

$$v = \frac{dx}{dt} = k(a - x)$$

In this equation v is the reaction velocity; a stands for the concentration of substance at the start of the reaction; x the substance changed in a given time t; and k represents a constant called the monomolecular reaction constant. If this is integrated,

$$k_k = \frac{1}{t} \ln \frac{a}{a - x}$$

is obtained.

The monomolecular law, however, has not been found to apply to enzymic reactions except under very limited conditions, as, for example, the first portion of the time course of the reaction, or within only a very limited range of initial concentrations. Nelson's (15) precise studies on invertase illustrate the failure of the monomolecular law to hold as conditions are varied. Other examples in which the monomolecular law holds only under certain restricted conditions are to be found in the work of von Euler (16) and Dernby (17), and in individual chapters to follow.

Effect of Enzyme Concentration on Reaction Velocity

Schütz (18) found that the amount of egg albumen hydrolyzed to peptone by pepsin in a given time with different amounts of pepsin was proportional to the square root of the concentration of pepsin. Northrop (19), however, showed that the products of proteolysis present in impure preparations of pepsin combine with the pepsin at the higher concentrations of enzyme to form enzymically inactive compounds. In purified preparations of pepsin, the Schütz law was not followed; instead the reaction velocity as determined by the reciprocal of the time necessary for a given extent of action was directly proportional to the concentration of enzyme. The rule that the reaction velocity is directly proportional to the enzyme concentration holds very widely. As O. Bodansky (20) has shown in the case of phosphatases this proportionality may not always be demonstrable unless optimal concentrations of activators are present.

Equation of Northrop

Northrop (19) has formulated an expression for the complete time course of peptic hydrolysis. The action he found was due to free pepsin, and the pepsin, after a few minutes, is inversely proportional to the amount of product. The pepsin may be present in solution free or in combination with the products of hydrolysis, the relative concentrations following the mass law. The following is Northrop's equation:

$$K = \frac{A \ln \frac{A}{A - x} - x}{ET}$$

where A is the original total concentration of substrate;

x is amount transformed at time T;
E is concentration of enzyme.

Northrop's equation represents the experimental results for the hydrolysis of albumen better than did Schütz's equation:

$$K = \frac{x}{\sqrt{ET}}$$

for the time course of the reaction.

According to Falk and associates (21), who studied the kinetics of tissue lipase, the monomolecular reaction velocity equation, Schütz's equation, and Northrop's equation, for this enzyme, were similar to those obtained with peptic digestion.

Michaelis-Menten Theory; Activity *pS* Curve

In 1913, Michaelis and Menten (22, 23) explained the action of invertase on sucrose by assuming that the initial velocity of hydrolysis, at different concentrations of sucrose, was proportional to the concentration of an intermediate sucrose-invertase compound

$$\frac{\psi}{\phi} = \frac{S}{S + K_s}$$

in which ψ represented the combined invertase, ϕ the total invertase, S the concentration of free sucrose, and K_s the dissociation constant of the intermediate compound. By considering the maximum initial velocity obtained (at about 5 per cent sucrose concentration) as 1, and initial velocities at lower sucrose concentrations as fractions of this, Michaelis and Menten were able to compare the experimental values shown in Fig. 4 with those given by the theoretical mass-law curve. As can be seen, the agreement between theory and experiment was satisfactory at concentrations of sucrose below 5 per cent. Curves like this, since they show the relation between the activity and the negative logarithm of the sucrose concentration, are known as *pS*-activity curves. The concept of Michaelis and Menten has been widely applied to other enzymes.

In recent years further light has been thrown on this theory. Nelson and Larson (24, 25), using phosphates and citrates, and a series of sucrose solutions having different H ion concentrations, were unable, in many experiments, to find any close harmony between the experimental and theoretical *pS*-activity curves. Nelson and Schubert (26) have explained the deviation of experimental values from

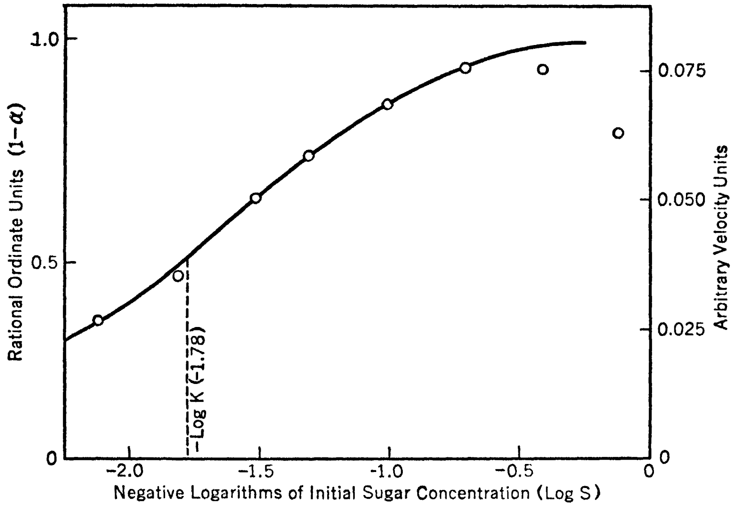


FIG. 4.—Comparison of the mass-law curve with the change in activity of yeast invertase as the concentration of sucrose is varied

the theoretical curve at high concentrations of sucrose by showing that at these concentrations of sucrose the concentration of water is of primary influence on the velocity of hydrolysis. These authors varied the concentrations of water and sucrose by adding given quantities of alcohol to the hydrolyzing sucrose solutions. Recently Stern (6*a*) has demonstrated, by spectroscopic means, the formation of an intermediate compound in the decomposition of monoethyl hydrogen peroxide by liver catalase. This intermediate compound exhibited the properties that had been postulated by Michaelis and Menten for an enzyme-substrate compound. The experiments of Stern will be discussed in the chapter on catalase.

Kinetics of Hydrolysis by Crude and Crystalline Trypsin

The digestion of casein by crude trypsin (formation of non-protein nitrogen) follows closely the course of monomolecular reaction, whereas with purified trypsin the velocity constant decreases as the reaction proceeds (27, 28, 29). This is more pronounced with a dilute substrate than with a concentrated one. With crystalline trypsin the values of the constant for the 2.5 and 5 per cent casein solution approach each other rapidly.

General Rule for Expressing Enzyme Activity

The method employed should yield results which are independent of the concentration of the enzyme solutions. With most enzymes the amount of change produced by the enzyme is only proportional to the enzyme concentration in the early part of the reaction. The potency of an enzyme is expressed in units. The unit of activity is the amount of change or destruction of a given quantity of substrate under certain definite conditions. Thus the specific activity (purity) of an enzyme preparation is measured by the number of activity units per gram of dry weight. Northrop uses the number of units per milligram of nitrogen.

Activators and Inhibitors

Non-specific substances which increase activity are called activators, e.g., HCl (pepsin), NaCl (amylase); and specific substances like cozymase (yeast, lactic acid), "coenzymes." "Toxic" substances like salts of heavy metals and certain organic compounds are called inhibitors (alkaloids, HCN, CO). The study of inhibitions (by heavy metals, oxidizing and reducing agents) led to the discovery of active groups in enzyme (urease, papain) molecules. The inhibitory (toxic) effect of certain inorganic salts like NaF upon enzymes is not yet quite explained. HCN inhibits the action only of some of the enzymes; others are not affected or are activated (papain, cathepsin).

Antiseptics. Enzymes, being proteins, are exposed to the destructive influence of microorganisms. A number of antiseptics have been tried, such as toluol, chloroform, sodium fluoride, phenol, thymol, and formaldehyde. Most of these have to a certain extent a destructive or an inhibitive effect on enzymes. For most purposes toluol is suitable. It can be separated by filtration from enzyme solutions. Dilute solutions of pepsin and trypsin, however, are destroyed by toluol (30), and liver esterase is inhibited by it (31). The effect of various antiseptics on enzymes has been discussed by Waksman and Davison (32). Extensive studies concerning this problem, however, are not available.

Kinetics of Inhibition

Studies based on the kinetics of enzymes lead to the assumption that there are definite enzymatically active chemical groups in the protein-enzyme molecule. It is suggested from the kinetics of enzymes, and from the chemical and stereochemical specificity, that unstable intermediate compounds form by combination of an active group with the substrate. The products of catalysis or other added substances may also form complexes with the active catalytic groups, resulting in a series of reversible equilibria. However, many of these combinations are irreversible. That this probably is true has been shown by Michaelis and Menten (22) and other workers (33).

For instance, esterases (including lipases) have been found to combine with a variety of substances such as alkaloids (34), chloroform, iodoform, formaldehyde (35), ketones, ketocarboxylic acid esters (36), and secondary alcohols (37). These substances inhibit apparently by the formation of enzyme-inhibitor complexes, by reducing the available enzyme concentration for combination with the substrate. These complexes are reversible. See also references 38 and 39. The stereoisomeric specificity of esterases (37, 40) definitely indicates certain optically active groups by which such combinations are brought about. Falk (41) furnished some indirect evidence concerning the active group in esterase, suggesting an enolic structure. This, however, is insufficient for a conclusion concerning the active group in this enzyme.

Inhibition Number

Glick and King (42) published a simplified expression for the inhibitory powers of various substances. These investigators introduced the term " inhibition number," and defined it as the ratio of the number of mols of methyl alcohol needed to produce 25 per cent inhibition (under the conditions of the experiment) to the number of mols of another substance needed to produce the same inhibition under similar conditions. Thus it takes (Table III) 436×10^{-6} mol of methyl alcohol to produce 25 per cent inhibition, whereas 143×10^{-6} molecule of ethyl alcohol is necessary to cause the same inhibition. Hence 436 ÷ 143

TABLE III

INHIBITION OF SHEEP LIVER ESTERASE BY NORMAL PRIMARY ALCOHOLS

Alcohol	Mols $\times 10^{-6}$ for 25 per cent inhibition	Inhibition number
Methyl	436.0	1.0
Ethyl	143.0	3.1
Propyl	53.0	8.2
Butyl	37.5	11.6
Amyl	9.0	48.4
Hexyl	4.9	89.0
Heptyl	1.8	242.0
Octyl	0.9	459.0
Nonyl	0.5	838.0

or 3.05 is the inhibition number of ethyl alcohol. The magnitude of the inhibition number, therefore, is a direct measure of the inhibiting power of a substance as compared to methyl alcohol, which is the standard, and original curves do not have to be consulted. The inhibition produced by normal primary alcohols on sheep liver esterase increases rapidly as the length of the hydrocarbon chain is increased.

Glick and King (42) showed that the inhibitions produced by the seven isomers of amyl alcohol decrease as the steric hindrance about the OH group increases. With secondary alcohols the effect of the size of the hydrocarbon group was greater than that of the nominal steric hindrance. The velocity of saponification was measured by continuous titration with 0.01 N NaOH, maintaining constant *p*H, using a buffer-free ethyl butyrate solution as a substrate according to the method of Knaffl-Lenz (43).

Recent work on activators and inhibitors has been reviewed by the author (44) and is presented in individual chapters that follow.

Reversible Inactivation

In the following, two examples of reversible inactivation of enzymes will be given. Here the *p*H and temperature are the influential factors. Kunitz and Northrop (45) inactivated *crystalline trypsin* solutions at various *p*H's and temperatures below 37°. They found that trypsin may be reversibly or irreversibly inactivated. The reversible inactivation occurs whenever the reversible denaturization of the protein is brought about. The denatured protein is in equilibrium with the native protein. An increase in temperature or alkalinity shifts the equilibrium towards the denatured side. Below *p*H 2.0, trypsin protein is changed into an inactive state, which may be irreversibly denatured by heat. This is a unimolecular reaction. The velocity increases with acidity.

Trypsin protein is slowly hydrolyzed between *p*H 2.0

and 9.0. This reaction is bimolecular, and the inactivation increases with increase in *p*H to 10.0, when it decreases. At *p*H 2.3 there is maximum stability. At *p*H 13.0 there is also formation of inactive protein; this reaction is unimolecular. With increasing *p*H, the velocity increases. From *p*H 9.0 to 12.0, some of the protein is hydrolyzed and some inactive protein is formed. At *p*H 13.0 inactivation is at a minimum. Decrease in activity was always proportional to decrease in tryptic protein. The rapid inactivation at higher temperature or in alkaline solutions is reversible for a short period only. The longer the solutions stand, the greater the loss in activity and irreversibility is manifested. Figure 5 shows inactivation at various *p*H's at 30° C. and 15° C.

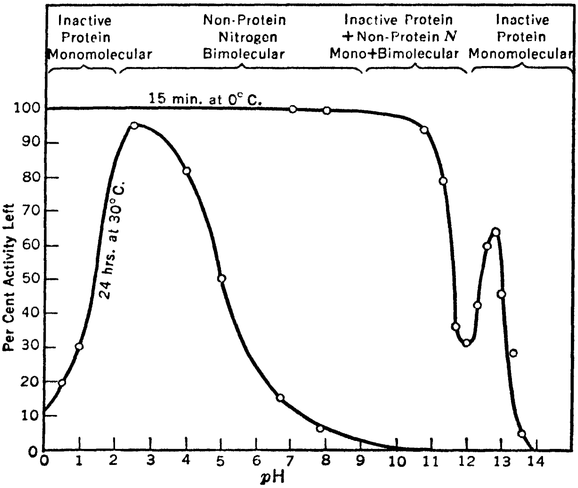


FIG. 5.—Loss in tryptic activity at various *p*H's

Pavlov and Parastschuk (46) found that alkali-inactivated pepsin may be reactivated when kept in a slightly acid solution for several hours. This has been confirmed by Northrop (47), using solutions of crystalline pepsin. He showed that pepsin solutions which have been completely denatured and inactivated by alkali (*p*H 10.5) could be reactivated by adjustment to about *p*H 5.4 and allowing to stand for twenty-four hours at 22° C. The reactivated material has been recrystallized and had the same peptic activity as the original pepsin.

The procedure of Pavlov and Parastschuk was followed by Anson and Mirsky (48, 49) for the reversal of protein denaturation.

Chemical Nature of Enzymes

The study of only the effects of biological substances does not satisfy the chemist. His aim is to analyze all compounds, as soon as isolated, and elucidate their structure so that synthesis may be effected as soon as possible. The properties (physiological as well as chemical) of many hormones and alkaloids have long been known before analysis was completed and before synthesis could be carried out. However, the discovery of certain biologically important substances has soon been followed by their preparation *in vitro*. Good examples are the recent isolation and synthesis of vitamin C (ascorbic acid), and vitamin B_2 (lactoflavin).

Enzyme action has been known for many years. In some instances efforts to isolate enzymes as pure chemical compounds have been successful. The early results, as well as the recent ones, may be divided into two groups. In one group it was shown that the enzymes are proteins, and in the other these same enzymes were considered to be non-protein substances. It will be seen that many of these reports have been based on erroneous conclusions, and it is indisputable now that many enzymes are proteins. Many investigators attempted to separate the protein from the active portion of the enzyme. This retarded the advancement in our knowledge of the chemical nature of enzymes.

In 1861 Brücke (50) obtained a pepsin preparation which did not give any protein color tests but gave a strong odor, resembling that of burning hair, when heated on platinum. Similar results were reported by Sundberg in 1885 (51). He obtained his pepsin by extracting calf's stomach mucosa with saturated NaCl solution and autolyzing the extract for several days. Then he precipitated the pepsin with calcium phosphate and redissolved it in HCl. After dialysis the *pepsin solution* lost most of its activity, but gave no protein color tests. In 1902 Pekelharing (52) obtained a very active pepsin preparation which he identified as a protein. His work was confirmed by many and served as a basis for the preparation of crystalline pepsin (see below). Pekelharing was the first who spoke of the possibility of crystallizing enzymes, being very enthusiastic about his highly refractive globules of pepsin. In 1930 Northrop announced the preparation of a crystalline albumin having peptic activity and being identical with the enzyme pepsin. This work has since been confirmed by several investigators (53, 54).

Rennin as prepared by the adsorption method has been found to contain only 0.678 per cent nitrogen (55). It will be seen that rennin is a thioproteose, having all the properties of such proteins (Tauber and Kleiner, and Holter).

Several *tryptases* which are proteins have been obtained in crystalline form (Northrop and Kunitz). One, however, has been obtained which is probably a polypeptide (Tauber and Kleiner, Willstätter and Rohdewald).

Pancreatic amylase of high activity has been obtained in crystallized form by Caldwell, Booher, and Sherman (1931), and identified as a protein. Waldschmidt-Leitz and Reichel (56), however, claim that this enzyme may be separated from protein, confirming the earlier work of Willstätter, Waldschmidt-Leitz, and Hesse (see Chapter VI). The dry weight of their preparation, however, indicates that the enzymé solution was too dilute to give protein color tests.

Malt amylases have also been found to be proteins. Denaturation of the enzyme protein was proportional to loss of activity (Sherman and associates). The chemical nature of *invertase* has been extensively studied by von Euler and Josephson (57, 58) and shown to be a protein.

This has been contradicted, however, by Willstätter and Kuhn (59).

Catalase has been shown to be a *hemin* containing chromoprotein, and the "*yellow enzyme*" (Warburg and Christian) a protein-pigment complex.

Urease, the first enzyme to be obtained in crystalline form, is a globulin (Sumner, 1926). *Pancreatic lipase* too has been found to be a globulin (Glick and King), and liver esterase appears to be an albumin (Sobotka and Glick). The chemical nature of these and other enzymes will be discussed in detail in individual chapters.

The Carrier Theory

In 1932 Willstätter and Rohdewald (60) repeated the early experiments of Brücke and Sundberg on the preparation of protein-free pepsin solution. They too found that the final *solutions* had only a very slight activity, but these solutions gave negative protein color tests. The author (61) has shown that diluted active enzyme solutions may easily escape the protein color tests, thus leading to the erroneous interpretation that enzymes are non-protein substances. For example, a solution of crystalline pepsin containing 0.1 mg. of the enzyme per cc., and able to clot 80 cc. of milk (*p*H 6.2) in ten minutes at 37°, gave a negative biuret and xanthoproteic test.

Willstätter and Rohdewald (60) do not believe that Northrop's protein crystals are identical with the enzyme pepsin or that any other enzyme might be a protein. Willstätter has been a firm supporter of the carrier theory, which was proposed by Perrin (62) in 1905. Generally by a carrier is meant any colloid of high molecular weight, such as a protein or a carbohydrate, which carries the active enzyme. This, however, is some substance of yet unknown chemical nature. The carrier is exchangeable for another carrier. Willstätter and Rohdewald have now modified the carrier theory. They state that enzymes have a " necessary colloidal carrier," whose chemical nature is still unknown.

Fodor (63) is also supporting the carrier theory. According to him the proteins are only the exchangeable carriers of the enzymes and are replaceable by any other colloid of a high molecular weight. The active principle of an enzyme has never been isolated, and there is little hope that it ever will be.

Glutathione acts in every respect like an enzyme; were its aqueous solution inactivated on boiling, it would be classified as an enzyme. Then, according to the carrier theory, the tripeptide would be only the carrier of the active principle.

Experiments have recently been published which were supposed to demonstrate that the carrier of pepsin (" pepsin-protein ") may be exchanged for other proteins (64, 65). It has been shown, however, that the peptic activity absorbed was equal to the uptake of pepsin protein (66, 67). Neither can the protein carrier of rennin be exchanged for another protein (68).

Digestion and Inactivation of Enzymes by Proteases

The carrier theory, which is based on theoretical assumptions only, has been found to be untenable. The digestibility of enzymes by proteases has been used extensively and appears to throw light upon this problem. A great many enzymes have been found to be digested by proteases (Table IV), and some with amazing rapidity. A fairly concentrated rennin solution is 90 per cent digested within six hours by a weak pepsin solution (*p*H 2.3), and a trypsin solution will cause the same degree of digestion in three hours (*p*H 6.2) (68). Most of these enzymes have been described as " free of protein." In view of their digestibility, their inactivation must be attributed to the digestion of their protein carrier. Is the carrier, if it really exists, always or almost always a protein? This would be contrary to the basic principles of the carrier theory, i.e., that the carrier may be *any* colloid of high molecular weight. So much has been written in favor of this theory that at times it was applied to explain non-enzymic problems. For example, Tillmans and associates (69), who contributed much to the isolation of vitamin C, thought that the reducing substance is the carrier of vitamin C. Now it is known that the reducing substance is vitamin C or ascorbic acid as it has been named.

TABLE IV

Digestion of Enzymes by Proteases

The digested enzyme	Protease employed for digestion	Reference
Sucrase	Trypsin	Von Euler and Josephson (70)
Urease	Trypsin, pepsin, papain	Tauber and Kleiner (71); Sumner, Kirk, and Howell (72)
Maltase	Trypsin	Tauber and Kleiner (73)
Rennin	Trypsin, pepsin	Tauber and Kleiner (68)
Pepsin	Trypsin	Tauber and Kleiner (74)
Lipase	Trypsin	Falk (75)
Trypsin	Pepsin	Long and Johnson (76); Long and Hull (77); Northrop and Kunitz (78); Tauber and Kleiner (74)
Salivary amylase	Trypsin, papain	Tauber and Kleiner (74)
Ascorbic acid oxidase	Trypsin	Tauber, Kleiner, and Mishkind (79)

Views Concerning the Mechanism of Enzyme Action

Although enzymes are catalysts, they differ from the so-called true or ideal catalysts (Wilhelm Oswald) in various ways. After the reaction, enzymes do not remain unchanged. Soon after the action commences a certain amount of destruction takes place, even at low temperature. The reaction velocity is not always proportional to the concentration of the enzyme. Enzymic catalysis can be reversed (synthesis) only in a few exceptional cases (proteins, organic esters, and carbohydrates). In contrast to inorganic catalysts (as HCl), only a few enzymes and these only under certain conditions follow the course of a monomolecular reaction. Thus the study of mechanism began, resulting in some very interesting findings.

According to the theory of Bayliss (80), enzyme action is based on an adsorption process, caused by the colloidal state of the enzyme; i.e., the enzyme adsorbs the substrate and then the chemical reaction takes place at the interface. This reaction may be explained by the law of mass action; the amount of adsorbed substance, however, is the controlling factor.

The Michaelis school believes that in certain reactions there is a combination between enzyme and substrate. Michaelis, too, applied the mass-action law for enzymic reactions, showing that the amount of combination between enzyme and substrate depends upon their concentrations.

It has been stated elsewhere that Northrop found that with pepsin and trypsin there is no combination between enzyme and substrate. The reaction takes place with the ionized part of the substrate. He also found that the reactions proceed (with slight deviations) according to the law of mass action, but there is a reversible combination between the enzyme and the reaction products.

Fodor (63) too believes that there is not enough evidence to assume a combination between enzyme and substrate as caused by specific groups and that the mass-action law cannot be applied to enzymic reactions. He is justified in so far as an enzyme-substrate complex has not yet been isolated. It has already been mentioned, however, that Stern (6*a*) has furnished experimental evidence for the existence of a complex in the case of catalase.

Synthesis by Enzymes

Under certain definite conditions reactions for some enzymes may be reversed. Wasteneys and Borsook (81) have been able to resynthesize 39 per cent of a peptic digest of egg albumin. Rona and associates have extensively studied the enzymic formation of esters (82, 83, 84). Especially interesting is the very rapid formation of *d* and *l* lactic acid esters of amyl alcohol by pancreatic esterase of the pig (85). See also Chapters II and V.

Preparation of Enzyme Material

The adsorption method was introduced by early investigators. It has been improved and is still extensively used by the Willstätter school. It is based on separation of the enzyme from extracts by adsorption on a suitable colloid such as kaolin and certain hydroxides of aluminum, and the subsequent elution (freeing) from the adsorbent. The adsorbent holds the enzyme by a small number of its affinities, and it can be released by mild agents such as weak alkalis or phosphates. By repeating this procedure a concentration of the enzyme takes place. This purification is based on the greater affinities of the adsorbent for the enzyme than for the impurities. The object of Willstätter and associates was to isolate a pure enzyme by this procedure. In this they did not succeed, but they have separated several enzyme mixtures into their components (see pancreatic enzymes, intestinal enzymes, and others). The adsorption procedure, however, is sometimes the cause of enormous loss in active enzyme material. For instance, an attempt to purify yeast invertase resulted in loss of 91 per cent of active enzyme. This was a large-scale operation starting with 9 kilos of brewer's yeast (Willstätter and Kuhn).

Cotton exerts selective adsorption toward enzymes (86). Here no contamination of the enzyme material takes place, as does with such adsorbents as inorganic gels, tannin, etc. Adsorption by cotton may be useful in testing the purity of crystalline enzymes.

For the *extraction* of enzymes sometimes a destruction of cellular matter may be necessary, i.e., chopping, grinding with sand, freezing followed by thawing, autolysis, separation of the cell juice by great pressure, or desiccation of the fresh tissue. The material which has been preliminarily treated can be extracted with dilute alcohol, dilute acetone, dilute glycerol, dilute acids, and alkalis, respectively. These extracts when treated with an equal volume of alcohol or acetone often yield quite active precipitates which may be dried in vacuum. Alcohol and acetone, however, are destructive to some enzymes, so that quick work is desirable. More concentrated preparations may be obtained by fractional isoelectric precipitation, i.e., isolating the most active fraction (see rennin). The fractional "salting-out" method is often used.

REFERENCES

1. Bechhold, H.: Colloids in Biology and Medicine. New York, 1919.
2. Tauber, H.: The chemical nature of enzymes. *Chem. Rev.*, **15**, 99 (1934).
3. Krebs, H. A.: Urea formation in the animal body. *Ergebnisse Enzymforschung*, **3**, 247 (1934).
4. Quastel, J. H., and Wooldridge, W. R.: The effects of chemical and physical changes in environment on resting bacteria. *Biochem. J.*, **21**, 148 (1927).
5. Szent-Györgyi, A. von: Non-enzymic catalysts of cellular oxidation. *Arch. exptl. Zellforschung*, **15**, 30 (1934).
6. Arrhenius, S.: Über die Reaktionsgeschwindigkeit bei der Inversion von Rohrzucker durch Säuren. *Z. physik. Chem.*, **4**, 226 (1889).

6*a*. Stern, K. G.: On the mechanism of enzyme action. A study of the decomposition of monoethyl hydrogen peroxide by catalase and of an intermediate enzyme-substrate compound. *J. Biol. Chem.*, **114**, 473 (1936).

7. Kastle, J. H., and Lowenhart, A. S.: Lipase, the fatsplitting enzyme, and the reversibility of its action. *Am. Chem. J.*, **24**, 491 (1900).
8. Lintner, C. J., and Kröber, E.: Zur Kenntniss der Hefeglycase. *Ber.*, **1**, 1050 (1895).
9. Luers, H., and Wasmund, W.: Über die Wirkungsweise der Amylase. *Fermentforschung*, **5**, 169 (1921–22).
10. Quastel, J. H., and Whetham, M. D.: The equilibrium existing between succinic, malic and fumaric acids in the presence of resting bacteria. *Biochem. J.*, **18**, 519 (1924).

11. Compton, A.: L'indépendance de la température optimale d'une diastase, à l'égard de la concentration en substrate et en diastase. *Ann. Inst. Past.*, **28**, 866 (1914). *Idem:* Studies in the mechanism of enzyme action. I. Rôle of the reaction of the medium in fixing the optimum temperature of a ferment. *Proc. Roy. Soc.*, **92**, 1 (1920–21).
12. Bodansky, A.: A study of a milk-coagulating enzyme of *Solanum elaeagnifolium*. *J. Biol. Chem.*, **61**, 365 (1924).
13. Howell, S. F., and Sumner, J. B.: The specific effects of buffers upon urease activity. *J. Biol. Chem.*, **104**, 619 (1934).
14. Caldwell, M. L., and Doebeling, S. E.: A study of the concentration and properties of two amylases of barley malt. *J. Biol. Chem.*, **110**, 739 (1935).
15. Nelson, J. M.: Enzymes from the standpoint of the chemistry of invertase. *Chem. Rev.*, **12**, 1 (1933).
16. Euler, H. Von: Fermentative Spaltung von Dipeptiden. *Z. physiol. Chem.*, **51**, 213 (1907).
17. Dernby, K. G.: Studien über die proteolytischen Enzyme der Hefe und ihre Beziehungen zu der Autolyse. *Biochem. Z.*, **81**, 107 (1916–17).
18. Schütz, E.: Eine Methode zu Bestimmung der relativen Pepsin Menge. *Z. physiol. Chem.*, **9**, 577 (1885).
19. Northrop, J. H.: The effect of the concentration of enzyme on the rate of digestion of proteins. *J. Gen. Physiol.*, **2**, 471 (1920).
20. Bodansky, O.: The accelerant effect of α-amino acids on the activity of bone phosphatase. *J. Biol. Chem.*, **114**, 273 (1936); *ibid.*, **115**, 101 (1936).
21. Sugiura, K., Noyes, H. M., and Falk, K. G.: Studies on enzyme action. XXIV. The kinetics of the ester-hydrolyzing actions of some tissue and tumor extracts. *J. Biol. Chem.*, **56**, 903 (1923).
22. Michaelis, L., and Menten, M. L.: Die Kinetic der Invertinwirkung. *Biochem. Z.*, **49**, 333 (1913).
23. Kuhn, R., and Münch, H.: Über Gluco- und Fructosaccharase. *Z. physiol. Chem.*, **163**, 3 (1926–27).
24. Larson, H. W.: Dissertation, Columbia University, 1927.
25. Nelson, J. M., and Larson, H. W.: Kinetics of invertase action. *J. Biol. Chem.*, **73**, 223 (1927).
26. Nelson, J. M., and Schubert, M. P.: Water concentration and the rate of hydrolysis of sucrose by invertase. *J. Am. Chem. Soc.*, **50**, 2188 (1928).
27. Northrop, J. H.: The kinetics of trypsin digestion. *J. Gen. Physiol.*, **6**, 417 (1924); *ibid.*, **16**, 295, 339 (1932).
28. Merrill, H. B.: The effect of enzyme purity on the kinetics of tryptic hydrolysis. *J. Gen. Physiol.*, **10**, 217 (1926).

29. Schönfeld-Reiner, R.: Vergleichende Untersuchungen über die Spaltbarkeit von Peptonen und Polypeptiden durch die Fermente der Pankreasdrüse. *Fermentforschung*, **12**, 67 (1930).
30. Effront, J.: Biochemical Catalysts in Life and Industry. Proteolytic Enzymes. Translated by Prescott, S. C., John Wiley & Sons, New York, 1917.
31. Glick, D., and King, C. G.: Relationships between the structure of organic compounds and their inhibiting effect upon liver esterase. Resemblance to a lyotropic series of anions. *J. Biol. Chem.*, **95**, 477 (1932).
32. Waksman, S. A., and Davison, W. G.: Enzymes. Williams & Wilkins Co., 1926.
33. Haldane, J. B. S.: Enzymes. Longmans, Green & Co., 38–49, 80–92, 1930.
34. Rona, P., and Pavlovic, R.: Beiträge zum Studium der Giftwirkungen. *Biochem. Z.*, **130**, 225 (1922).
35. Palmer, L. S.: The influence of various antiseptics on the activity of lipase. *J. Am. Chem. Soc.*, **44**, 1527 (1922).
36. Willstätter, R., Kuhn, R., Lind, O., and Memmen, F.: Über Hemmung der Leberesterase durch Ketocarbonsäureester. *Z. physiol. Chem.*, **167**, 303 (1927).
37. Murray, D. R. P., and King, C. G.: The stereochemical specificity of esterases. I. The affinity of liver esterases for optical active alcohols. *Biochem. J.*, **24**, 190 (1930).
38. Weber, H. H. R., and King, C. G.: Specificity and inhibition characteristics of liver esterase and pancreas lipase. *J. Biol. Chem.*, **108**, 131 (1935).
39. Baker, Z., and King, C. G.: The purification, specificity and inhibition of liver esterase. *J. Am. Chem. Soc.*, **57**, 358 (1935).
40. Rona, P., and Ammon, R.: Die stereochemische Specifität der Esterasen und die synthetisierende Wirkung der esterspaltenden Fermente. *Ergebnisse Enzymforschung*, **2**, 50 (1933).
41. Falk, K. G.: The action of alkali in the production of lypolytically active protein. *Proc. Nat. Acad. Sci.*, **2**, 551 (1916).
42. Glick, D., and King, C. G.: Relationships between the structure of saturated aliphatic alcohols and their inhibiting effect upon liver esterase. *J. Biol. Chem.*, **94**, 497 (1931).
43. Knaffl-Lenz, E.: Über die Kinetic der Esterspaltung durch Leberlipase. *Arch. exptl. Path. Pharm.*, **97**, 242 (1923).
44. Tauber, H.: Activators and inhibitors of enzymes. *Ergebnisse Enzymforschung*, **4**, 42 (1935).
45. Northrop, J. H.: Inactivation of crystalline trypsin. *J. Gen. Physiol.*, **17**, 591 (1934).
46. Pavlov, J. P., and Parastschuk, S. W.: Über die ein und demselben

Eiweissfermente zukommende proteolytische und milchkoagulierende Wirkung verschiedener Verdauungssäfte. *Z. physiol. Chem.*, **42**, 415 (1904).

47. NORTHROP, J. H.: Crystalline pepsin. III. Preparation of active crystalline pepsin from inactive denatured pepsin. *J. Gen. Physiol.*, **14**, 713 (1931).
48. ANSON, M. L., and MIRSKY, A. E.: On some general properties of proteins. *J. Gen. Physiol.*, **9**, 169 (1925–26).
49. ANSON, M. L., and MIRSKY, A. E.: Protein coagulation and its reversal. Serum albumin. *J. Gen. Physiol.*, **14**, 725 (1931).
50. BRÜCKE, E.: Beitrage zur Lehre von der Verdauung. *Sitzber. Akad. Wiss. Wien, Math. naturw. Klasse*, **43**, 601 (1861).
51. SUNDBERG, B.: Ein Beitrag zur Kenntniss des Pepsins. *Z. Physiol.*, **9**, 319 (1885).
52. PEKELHARING, C. A.: Mitteilungen über Pepsin. *Z. physiol. Chem.*, **35**, 8 (1902).
53. HOLTER, H.: Über die Labwirkung. *Z. physiol. Chem.*, **196**, 1 (1931).
54. LOUGHLIN, W. J.: The heat inactivation of crystalline pepsin; the critical increment of the process. *Biochem. J.*, **27**, 1779 (1933).
55. LUERS, H., and BADER, J.: Über die Reinigung des Ghymosins. *Biochem. Z.*, **190**, 122 (1927).
56. WALDSCHMIDT-LEITZ, E., and REICHEL, M.: Zur Frage nach der chemischen Natur der Pankreasamylase. *Z. physiol. Chem.*, **204**, 197 (1932).
57. EULER, H. VON, and JOSEPHSON, K.: Saccharase. II. *Ber.*, **56**, 1096, 1907 (1923). Saccharase. III. *Ibid.*, **57**, 299 (1924).
58. EULER, H. VON, and JOSEPHSON, K.: Die Saccharase als amphoterer Elektrolyt und als Kolloid. *Z. physiol. Chem.*, **133**, 279 (1924).
59. WILLSTÄTTER, R., and KUHN, R.: Über Spezifität der Enzyme. *Z. physiol. Chem.*, **125**, 28 (1923).
60. WILLSTÄTTER, R., and ROHDEWALD, M.: Über Desmo-pepsin und Desmo-kathepsin. *Z. physiol. Chem.*, **208**, 258 (1932).
61. TAUBER, H.: The chemical nature of emulsin, rennin and pepsin. *J. Biol. Chem.*, **99**, 257 (1932).
62. PERRIN, J.: Un modele de diastase. *J. chim. phys.*, **3**, 102 (1905).
63. FODOR, A.: Mechanismus der Enzymwirkung. *Ergebnisse Enzymforschung*, **1**, 39, (1932).
64. DYCKERHOF, H., and TAWES, G.: Über die Adsorption von Pepsin an Eiweiss. *Z. physiol. Chem.*, **215**, 93 (1933).
65. WALDSCHMIDT-LEITZ, E., and KOFRANYI, E.: Die komplexe Natur des "kristallisierten Pepsin," *Naturwissenschaften*, **21**, 206 (1933).
66. NORTHROP, J. H.: Absorption of pepsin by crystalline proteins. *J. Gen. Physiol.*, **17**, 165 (1933).
67. SUMNER, J. B.: Parallel adsorption of crystalline pepsin and peptic

activity on casein and ovalbumin. *Proc. Soc. Exptl. Biol. Med.*, **31**, 204 (1933).

68. Tauber, H., and Kleiner, I. S.: The chemical nature of rennin. *J. Biol. Chem.*, **104**, 259 (1934).
69. Tillmans, J., Hirsch, P., and Dick, H.: Das Reduktionsvermögen pflanzlicher Lebensmittel und seine Beziehung zum Vitamin C. *Z. Untersuch. Lebensm.*, **63**, 1 (1932).
70. Euler, H. von, and Josephson, K.: Saccharase. IV. *Ber.*, **57**, 859 (1924).
71. Tauber, H., and Kleiner, I. S.: Studies on crystalline urease. IV. The "antitryptic" property of crystalline urease. *J. Gen. Physiol.*, **15**, 155 (1931).
72. Sumner, J. B., Kirk, J. S., and Howell, S. F.: Digestion and inactivation of crystalline urease by pepsin and by papain. *J. Biol. Chem.*, **98**, 543 (1932).
73. Tauber, H., and Kleiner, I. S.: The digestion and inactivation of maltase by trypsin and the specificity of maltases. *J. Gen. Physiol.*, **16**, 767 (1933).
74. Tauber, H., and Kleiner, I. S.: The inactivation of pepsin, trypsin and salivary amylase by proteases. *J. Biol. Chem.*, **105**, 411 (1934).
75. Falk, K. G.: Studies on enzyme action. *J. Biol. Chem.*, **103**, 363 (1933).
76. Long, J. A., and Johnson, A.: On some conditions affecting the activity and stability of certain ferments. II. *J. Am. Chem. Soc.*, **35**, 1188 (1913).
77. Long, J. A., and Hull, M.: On the assumed destruction of trypsin by pepsin and acid. *J. Am. Chem. Soc.*, **38**, 1620 (1916).
78. Northrop, J. H., and Kunitz, M.: Crystalline trypsin. I. *J. Gen. Physiol.*, **16**, 267 (1932).
79. Tauber, H., Kleiner, I. S., and Mishkind, D.: Ascorbic acid (vitamin C), oxidase, *J. Biol. Chem.*, **110**, 211 (1935).
80. Bayliss, W. M.: The Nature of Enzyme Action. 5th Ed. London.
81. Wasteneys, H., and Borsook, H.: The enzymic synthesis of proteins. *Physiol. Rev.*, **10**, 110 (1930).
82. Rona, P., Chain, P., and Ammon, R.: Beiträge zur enzymatischen Esterbildung und Esterspaltung. *Biochem. Z.*, **247**, 8 (1932).
83. Rona, P., Ammon, R., and Trurnit, H. I.: Die enzymatische Bildung von Mandelsäurestern. *Biochem. Z.*, **247**, 100 (1932).
84. Rona, P., and Ammon, R.: Versuche über enzymatische Ester-Hydrolyse und Synthese. *Biochem. Z.*, **249**, 446 (1932).
85. Rona, P., and Ammon, R.: Asymmetrische Esterifizierung durch Schweinepankreasesterase. *Biochem. Z.*, **217**, 34 (1930).
86. Tauber, H.: The selective adsorption of enzymes by cellulose. *J. Biol. Chem.*, **113**, 753 (1936).

CHAPTER II

ESTERASES

The enzymic ester hydrolysis or ester synthesis proceeds according to the equation

$$R \cdot COOR' + H_2O \rightleftarrows R \cdot COOH + R'OH.$$

If in the above equation $R \cdot COOH$ is a higher fatty acid and $R'OH$ is glycerol, the enzyme responsible for the hydrolysis is lipase, e.g., pancreatic lipase, gastric lipase, ricinus lipase. If, however, $R \cdot COOH$ is any other organic acid or if the acid is an inorganic one and $R'OH$ a simple alcohol (aliphatic or aromatic) or a carbohydrate, then the reaction is catalyzed by an esterase, e.g., liver esterase, sulfatase, phosphatase, etc. The specificity of lipases and certain esterases, however, is not absolute, and the presence of an asymmetric carbon atom in the alcohol or acid radical also influences the specificity of the esterase.

Pancreatic Lipase

Pancreatic lipase may be obtained either directly from the pancreas gland or from the pancreatic juice. In accordance with the classification given above, pancreatic lipase hydrolyzes glycerides of the higher fatty acids but does not readily attack lower esters. Claude Bernard was the first to show (in 1856) that pancreatic juice has lipolytic activity. It was believed that the pancreatic juice contained two lipolytic enzymes: i.e., a " fat " (lipase) and a " low ester " (esterase) splitting enzyme. Later it was definitely established that there was only one such enzyme, the fat-hydrolyzing lipase present in the pancrease (1–3). This lipase hydrolyzes true fats very rapidly.

Preparation. Willstätter and Waldschmidt-Leitz (4) obtained active enzyme preparations by extracting acetone and ether dehydrated and defatted pig pancrease with water and glycerol. Apparently the acetone and ether did not affect this enzyme. Glick and King (5) employed 10 per cent NaCl for the extraction of lipase from dry pancreatic tissue. Their method was more suitable for concentrating the enzyme than those of any of earlier investigators. The lipase precipitates quantitatively on saturation with $MgSO_4$. Bamann and Laeverenz (6) reported on an accidental observation of a " crystalline lipase protein," which they did not study further.

Pancreatic Prolipase

The preparation of an inactive zymogen prolipase was first made by Rosenheim (7) in 1910. He demonstrated that prolipase is thermolabile and that it may be activated by serum and extracts of tissues. He showed that by diluting pancreatic extracts with water the prolipase separates as a precipitate and the coenzyme remains in solution. In 1932 Woodhouse (8) obtained some interesting results on the prolipase-activating power of various sera (see Table V) and inorganic compounds (see Table VI), using olive oil as a substrate. He also verified the earlier work of Rosenheim on prolipase. That certain inorganic salts may either activate or inhibit castor bean lipase was shown, however, by Falk (9), as early as 1913.

The activity of pancreatic and liver lipase is accelerated by certain concentrations of sodium taurocholate, and inhibited by greater concentrations of the salt (9*a*). Copper sulfate inhibits pancreatic and liver lipase but has no effect on serum lipase (10).

The Stereospecificity of Esterases

Dakin (11, 11*a*) noticed that esterases exert a selective action on a racemic mixture of the substrate. For instance,

TABLE V

ACTIVATION OF PROLIPASE BY VARIOUS SERA

Prolipase cc.	Activator cc.	Olive oil emulsion cc.	Titration cc. N/10 NaOH
0.5	....	5.0	1.2
0.5	1.0 coenzyme *	5.0	19.6
0.5	0.5 ox serum	5.0	32.7
0.5	0.5 rabbit serum	5.0	25.6
0.5	0.5 human serum	5.0	30.8
...	1.0 coenzyme *	5.0	0.85
...	0.5 ox serum	5.0	0.60

* Prepared by boiling equal volumes of aqueous pancreatic extract and water and filtering.

TABLE VI

EFFECT OF INORGANIC COMPOUNDS ON PROLIPASE

Prolipase cc.	Ox serum cc.	Salt 1 per cent solution cc.	Water cc.	Olive oil emulsion cc.	Titration cc. N/10 NaOH
...	...	1.0 lead acetate	1.0	4.0	0.7
0.5	...	....	1.5	4.0	1.2
0.5	0.5	....	1.0	4.0	26.8
0.5	0.5	1.0 lead acetate	0.5	4.0	33.4
0.5	...	1.0 lead acetate....	0.5	4.0	13.4
0.5	...	1.0 lead chloride	0.5	4.0	12.2
0.5	...	1.0 cuprous chloride	0.5	4.0	0.5
0.5	...	0.5 thallium acetate	1.0	4.0	0.5
0.5	...	1.0 uranyl acetate	0.5	4.0	1.8
0.5	...	1.0 cupric chloride	0.5	4.0	1.4
0.5	...	1.0 $Co(NO_3)_2$	0.5	4.0	14.8
0.5	...	1.0 $MnCl_2$	0.5	4.0	3.0

pig liver esterase added to *dl*-mandelic acid esters would hydrolyze the *d*-form first. If, however, another *dl*-ester was used, the *l*-form was first hydrolyzed. Pancreatic (pig) lipase hydrolyzed the *d*-form first when added to the racemic mixture of *dl*-mandelic ester, but when the *d*-form and *l*-form were split separately, the latter was hydrolyzed faster (12). Experiments of this sort are unlimited, since the number of esters is great. An extensive review of this subject has been given by Rona and Ammon (13).

Optimum *p*H

It is impossible to designate any specific optimum *p*H for pancreatic lipase, and a few other enzymes. Their optimum *p*H depends upon the nature of the enzyme preparation, the rate of hydrolysis (14), the buffer, and the substrate. The optimum *p*H of pancreatic lipase, for instance, with olive oil as a substrate and NH_3–NH_4Cl as a buffer, is at 9.2; with tributyrin, however, it is at 8.3 (4). When tripropionin is the substrate the optimum *p*H is at 7.2 with phosphate-borate, and 9.3 with glycine as a buffer (14).

Estimation

In the hydrolysis of olive oil, the fatty acids formed are titrated with alkali (5, 15), or hydrolysis of trybutyrin may be followed by the stalagmometric method (16–18*a*), or the histochemical method (19). These methods may be employed for the determination of all esterases.

Gastric Lipase

The earlier literature does not lack data concerning gastric esterase. Of the more recent papers the one by Willstätter and Memmen (20) should be mentioned. These workers prepared the enzyme by extracting the dry gastric mucosa with dilute alkali or with H_2O and glycerol respectively. This enzyme has an optimum *p*H of about 5 (trybutyrin). It does not hydrolyze fats readily. It does, however, hydrolyze emulsified fats such as the fat of cow's milk and of egg yolk. It is extremely sensitive to acid, which makes its detection in gastric juice very difficult (21).

Liver Esterase

Liver esterase differs from pancreatic lipase since it is a typical ester- (not fat-) splitting enzyme. It hydrolyzes readily esters of simple alcohols. For the earlier studies ethyl acetate and ethyl butyrate were employed (22, 23). The optimum of liver esterase is between *p*H 6.7 and 8.2, depending upon the buffer, the substrate, and the source of the enzyme (24).

Preparation. All kinds of procedures have been tried for the purification of liver esterase but without much success as to the concentration of the enzyme. Dakin (25) ground pig liver with some kieselguhr and sand, and centrifuged the hydraulic press juice. The juice when kept in the ice chest was quite stable. Pierce (26) obtained a purer preparation by extending the method of Dakin with dialysis and precipitation with an equal volume of saturated ammonium sulfate. The precipitate had considerable activity. Full saturation of the filtrate with ammonium sulfate yielded an inactive filtrate and an active precipitate. The latter was dialyzed until free of SO_4. The remaining solution was highly active. Kraut and Rubenbauer (27) state that they obtained by dialysis and adsorption a protein-free active liver esterase. See also Baker and King (28).

Induction Time. In hydrolysis of mandelic acid ethyl ester by liver esterase an induction time has been noticed. During the first hour or so there is a delay in the hydrolysis of the ester. No definite explanation can be given as yet (29). Other enzymes exhibit similar phenomena (see zymase).

Experiments Showing Differences Between the Liver and Pancreatic Enzyme

A Comparison of Relative Velocity of Hydrolysis. There is a distinct difference between the relative velocity of hydrolysis of pancreatic lipase and liver esterase (9*a*).

Pancreatic lipase is a powerful, fat-splitting enzyme and does not readily attack lower esters, whereas with liver esterase this is reversed.

Differences in Activation and Inhibition. Bile salts, and other organic compounds which activate pancreatic lipase greatly, exhibited strong inhibition toward liver esterase (30), though in higher concentrations pancreatic lipase was also inhibited (9*a*).

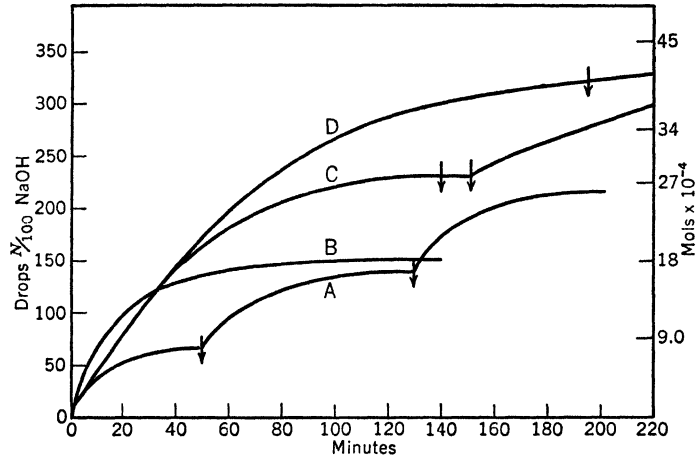


FIG. 6.—Kinetics of monobutyrin hydrolysis by pancreatic lipase. Curve *A*, hydrolysis of 0.9 mM monobutyrin in 50 cc. reaction volume at 28° by 0.1 cc. of pancreas lipase solution. Addition of 0.9 mM monobutyrin after 50 and 130 minutes. Curve *B*, hydrolysis of 1.8 mM monobutyrin under the same conditions. Curve *C*, hydrolysis of 3.4 mM monobutyrin in 50 cc. by 0.1 cc. of pancreas lipase. Addition of 0.1 cc. of pancreas lipase after 140 minutes and of 0.1 cc. of liver esterase solution after 150 minutes. Curve *D*, hydrolysis of 4.5 mM monobutyrin in 50 cc. (emulsion) by 0.1 cc. of pancreas lipase. Addition of 0.1 cc. of pancreas lipase after 195 minutes. Titration with 0.01 *N* NaOH according to Knaffl-Lenz. The horizontal lines at the right margin indicate 2 per cent hydrolysis of the total amounts of monobutyrin given.

Differences in the Reaction Course. The kinetics of liver esterase follows a linear or 0 molecular reaction, owing to the extremely high affinity for its substrate. This is true even after 90 per cent of the substrate is split, showing that the cleavage products do not inhibit. The kinetics of pancreatic lipase, however, are more complicated. There is at first a steep rise, followed immediately by an almost horizontal course (see Fig. 6). This has been explained by a consideration of the rôle of the inactive areas on the colloidal enzyme particles (30*a*; see also 14).

Ricinus Lipase

Ricinus lipase was discovered in 1890 by Green (31) in the germinating seeds of the castor bean (*Ricinus communis*). Green found that this plant enzyme is a typical lipase, hydrolyzing true fats, similar to pancreatic lipase. Since that time the observations of Green have been confirmed many times, and it was found that lower esterases are hardly attacked by this lipase (32). Its optimum *p*H is between 4.7 and 5.0 (33). It varies slightly according to the buffer employed. The enzyme may be readily extracted from the castor bean seeds, with dilute alkali. Methods for the purification have been described by a number of authors (33, 34, 34*a*).

Longenecker and Haley (34*a*) found that ricinus lipase showed no specificity in its attack on glyceride molecules containing carbon chains of different length. The number of mols of glyceride hydrolyzed was taken as a basis.

The castor bean contains toxic proteins. To avoid the danger of poisoning, the separation of the shells must be done with rubber gloves, and when working with dry enzyme preparations the powder should not be inhaled.

Chlorophyllase

Chlorophyllase is present in all green plants. It splits the alcohol phytol $C_{20}H_{39}OH$ from chlorophyll *a*, converting it to chlorophyllid *a* (35). The enzyme may be prepared free from chlorophyllid *a* by drying the leaves and extracting them with alcohol. Fats or waxes are not hydrolyzed by this enzyme. The reaction course of chlorophyllase follows an equation of the first order. The enzyme may be estimated colorimetrically (36). It was with the aid of this enzyme that Willstätter and Hug (37) isolated "crystalline chlorophyll" (ethylchlorophyllide).

Tannase

The enzymic hydrolysis of tannin was described by Scheele in 1786. Powerful preparations have been obtained from the mold *Aspergillus niger* (38, 39). Freudenberg (40) has obtained valuable information concerning the structure of various tannins by studying the action of tannase.

Dyckerhoff and Armbruster (41) have reported their ability to obtain tannase solutions free from esterases, by selective destruction of the esterases, at an alkaline *p*H. In contrast to other esterases tannase hydrolyzes only those esters which have an acid component containing at least two phenolic hydroxyl groups. None, however, can be ortho to the carboxyl group. The alcohol group does not affect the specificity. A direct combination of the ester-carboxyl to the oxidized benzene ring is necessary for an ester to be hydrolyzable by tannase. For the estimation of tannase activity the method of Rhind and Smith (42) may be used.

Sulfatase

Sulfatase was first observed by Neuberg (43). It hydrolyzes ethereal sulfates (sulfuric acid esters of phenols) which are products of detoxication of the human body. Sulfatase may be prepared from the mold *Aspergillus oryzae*, from muscles, and kidneys. The last is the best source (44).

Glucose and saccharose sulfuric acid esters are hydrolyzed by a specific chondro sulfatase present in bacteria, but are not split by kidney sulfatase (44*a*).

Phosphatases

Phosphatases play an important rôle in bone formation, muscle metabolism, lactation, and alcoholic fermentation. They hydrolyze phospholipids, phosphoproteins, phosphocreatine, phosphoarginine, phosphoric esters of carbohydrates, phosphoglyceric acids, glycerophosphoric acids, and nucleic acids.

Preparation and Estimation of Tissue Phosphatases. Active extracts may be prepared by adding 20 parts of water to 1 part of tissue and allowing autolysis to proceed for two to three days (Kay). The influence of magnesium ions on the determination of the activity of phosphatases has been noted by several investigators (Erdtman, Homerburg, Jenner and Kay, Bodansky and Bakwin). Studies under carefully controlled conditions have been published by O. Bodansky (44*b*), who has in addition observed the effect of α-amino acids on the course of hydrolysis of β-glycerophosphate. He has determined the optimal conditions for the estimation of tissue phosphatase activity.

The following tentative classification has been suggested by Foley and Kay.

I. Phosphomonoesterases. These esterases are found in various plant and animal tissues and are abundant especially in mammalian tissues. They readily split all monoesters of orthophosphoric acid but not disubstituted esters. Phosphomonoesterases are the most studied phosphatases and they may be grouped into four classes. (*a*) The " alkaline phosphatase " of bone, kidney, and intestine is best known. It has an optimum *p*H of 9 to 10 (45), this being the most alkaline *p*H yet observed for an enzyme. Kay (45) believes that this enzyme may be identical with nucleotidase and hexosediphosphatase (see also reference 46). This enzyme splits sodium β-glycerophosphate more readily than the α-salt (45). (*b*) A second phosphatase has been separated from the alkaline one, using extracts of certain mammalian organs by selective hydrogen-ion inactivation (47). The *p*H activity curve shows two peaks, one at *p*H 9.0 and one at *p*H 5.0 with a maximum at 9.0 (*c*). The third type of phosphomonoesterase has an optimum *p*H at 3.0 to 4.0. This enzyme is found in taka diastase (48) and *Aspergillus oryzae* (49). (*d*) The fourth type of phosphomonoesterase has an optimum *p*H at 6.5 and is present in red blood cells (50).

II. Phosphodiesterases. This group of enzymes splits only one of the two linkages in a diesterified orthophosphoric acid, and further action of a phosphomonoesterase is necessary for complete hydrolysis of the diester. Rice bran and snake venom are good sources of phosphodiesterases (51). Triesters of phosphoric acid are not hydrolyzed by phosphatases.

Various other phosphatases which are believed to be specific have been described, such as lecithinases, pyrophosphatases, phosphoamidases, and others. These have been discussed in a very extensive review by Folley and Kay (52).

Plasma or Serum Phosphatase in Disease. As a result of the intensive researches of several groups of investigators, it is a well-established fact now that the phosphatase content of human blood plasma is practically always increased in *bone diseases* such as rickets (infantile, renal, and adolescent), generalized osteitis fibrosa, osteomalacia, osteitis deformans, and osteoplastic bone tumors. The phosphatase increases with the severity of the disease (Kay, Roberts, Hunter, A. Bodansky and Jaffe, Smith, and others) (52).

A. Bodansky and Jaffe (53) in the course of a highly interesting clinical study found that serum calcium and inorganic phosphorus are not reliable criteria of the severity of rickets at admission or of the rate of healing of rickets. They found that in normal children the serum phosphatase range is between 5 and 15 units per 100 cc. It rises to 20 or 30 units in " mild " rickets, in " marked " cases it may be as high as 60 units, and in " very marked " cases it exceeds 60 units. These figures and clinical and roentgenologic evidence have independent value as bearing on different aspects of rickets, and complete each other as criteria of the severity of rickets at admission. The phosphatase value may also be used as a criterion of the effectiveness of the treatment. Even when therapy (viosterol, cod-liver oil) is " rapidly effective," a lag of four to twelve days may occur, according to A. Bodansky and Jaffe, before a marked decrease in phosphatase is manifested. The decrease continues rapidly until a high normal value is reached within two months or less (see Table VII).

In jaundice of the obstructive type plasma phosphatase is also increased (54), and by combining phosphatase determinations with bilirubin measurements obstructive jaundice may be differentiated from jaundice of toxic and catarrhal origin.

Tenner and Kay (54*a*) found an increase in plasma phosphatase in acute enteritis, in arthritis, in tuberculosis, and in certain bone diseases. The increase was very great in osteitis fibrosa cystica and Paget's disease.

In cancer erythrocytes have, in general, a higher phosphatase content over the *p*H range 5.0–6.0 (54*b*). An accurate method for the estimation of serum phosphatase has been devised by A. Bodansky (54*c*).

Choline Esterase

The existence of a choline esterase was first suggested by Dale (55). Acetylcholine is a physiologically important substance. It has a powerful effect upon the blood pressure (56, 56*a*, 57) and upon muscle contraction (58). It has been shown by Stedman, Stedman, and Easson (59, 59*a*) that the acetyl esterase content of the blood may be of diagnostic value in heart studies. These authors named this enzyme choline esterase. According to Feldberg (60), it protects the organism from acetylcholine poisoning. Choline esterase may be responsible for the short effect of acetylcholine (61, 62), and the synthesis of choline esterase-

TABLE VII

Serum Calcium, Inorganic Phosphorus, and Phosphatase in the Course of Treatment of Marked Infantile Rickets

			Very Marked Rickets		
Date	Calcium, Mg.	Inorganic Phosphorus, Mg.	Phosphatase Units	Healing	Treatment
1931		Case 1: Female, 4 years			
4/27	10.3	2.6	....	....	Viosterol (30)
5/13	....	3.5	58	....	
6/ 3	....	5.3	43	....	
		Case 2: Female, 3 years			
3/30	8.6	3.3	76	....	None
4/ 7	8.4	2.5	....	....	
4/13	....	...	....	....	Viosterol
4/27	10.2	3.9	37	++	
5/22	10.1	5.0	22	++	
1932 *		Case 3: Male, 17 months			
2/ 3	9.8	2.7	143	....	Viosterol (60)
2/11	....	3.2	107	....	
2/19	....	4.1	42	+	
2/25	....	4.0	37	....	
3/ 7	9.5	5.7	17	++	
3/23	9.9	5.5	14	....	
4/ 8	10.8	6.2	13	++	
4/20	....	5.9	14	....	Cod-liver oil (?)
9/28	10.8	4.9	13	....	
1/25	....	5.4	8	....	
		Case 4: Female, 21 months			
4/13	9.4	2.7	138	....	Cod-liver oil
4/26	....	3.7	135	....	
5/ 9	....	4.6	149	....	
5/12	10.1	4.3	150	+?	Viosterol (30)
5/17	....	4.4	139	....	
5/24	....	4.8	140	+	Viosterol (60)
5/31	....	5.9	107	....	
6/ 7	....	5.4	88	....	
6/14	....	4.7	58	....	Cod-liver oil
9/ 6	....	4.9	42	....	
		Case 5: Male, 2 years			
11/22	6.9	3.8	66	....	Ultra-violet rays, local
12/ 2	....	4.1	75	....	
12/14	9.1	4.0	77	+	
12/20	9.4	4.1	68	+	Ultra-violet rays, general
12/27	....	3.9	59	....	
1/ 4	....	4.4	54	++	
1/13	....	5.3	44	++	Viosterol (60)
1/25	....	5.9	32	+	
2/ 7	....	5.5	20	....	
2/16	....	6.8	22	....	

The available evidence of healing, from clinical and roentgenologic records, is summarised as follows: +, slow healing; ++, marked and rapid healing.

* In this and other cases, when the observations were carried into the following year, the year can be readily inferred from the other dates stated.

TABLE VII—*Continued*

Marked Rickets

Date	Calcium, Mg.	Inorganic Phosphorus, Mg.	Phosphatase Units	Healing	Treatment
1931	Case 6: Male, 2 years				
2/18	9.8	2.3	....	....	None
3/ 6	....	1.5	37.3	....	
3/25	10.2	1.9	55.6	....	Viosterol (60)
4/15	....	...	....	+	Viosterol (90)
4/27	10.6	4.7	15.4	....	
5/12	10.2	5.4	12.6	++	
	Case 7: Female, 13 months				
7/14	8.9	2.5	57.0	....	Viosterol ?
8/31	11.2	5.2	....	....	
9/22	....	5.5	7.2	....	
10/13	....	5.6	7.5	....	Cod-liver oil?
2/16	11.8	4.6	10.9	....	
	Case 8: Female, 2 years				
7/29	9.1	2.9	51.3	....	Viosterol irreg.
8/19	9.6	5.7	....	....	
8/28	10.4	3.8	....	....	
9/16	9.8	4.0	27.3	....	
10/ 9	....	6.0	17.5	++	
	Case 9: Male, 2 years				
10/24	9.8	3.4	31.8	....	Viosterol (60); cod-liver oil
11/ 6	....	3.8	21.3	....	
11/23	10.0	5.5	17.0	++	
11/25	....	...	....	....	Osteotomy; treatment continued
12/ 8	10.0	5.2	6.2	....	
1932	Case 10: Female, 2 years				
2/25	....	3.1	45.5	....	Viosterol (30)
3/ 7	10.7	5.3	31.3	+	
3/23	11.0	5.8	17.1	....	
4/ 8	10.7	4.8	12.6	++	
	Case 11: Female, 21 months				
5/31	8.5	2.5	49.0	....	
6/ 7	....	2.5	57.0	....	Viosterol irreg.
6/22	9.7	3.5	58.0	....	Viosterol (60)
6/28	....	4.5	54.0	....	
7/ 8	....	5.7	27.6	....	
7/21	10.4	5.8	19.0	....	
	Case 12: Male, 21 months				
6/18	9.3	3.0	43.8	....	Viosterol (60)
6/28	....	6.2	40.6	....	Cod-liver oil
9/29	....	5.4	18.2	....	
12/20	....	6.5	17.5	++	
4/13	....	6.0	11.9	....	
	Case 13: Male, 14 months				
6/23	10.0	2.9	51.5	....	Viosterol (60)
7/ 8	....	3.9	32.8	....	

resistant derivatives for pharmaceutical purposes is planned (63).

Choline esterase occurs in many tissues. Good sources are heart muscle, intestinal mucosa, and blood. A very good source is found in certain snails (*Helix pomatia*) (64). This esterase is specific since it does not parallel the lipase content of various organs (59*a*, 65). Table VIII shows choline esterase and esterase activities of various blood sera, indicating two specific enzymes (59*a*). For further details the review by Ammon (64) should be consulted.

Sym's Method for the Enzymic Synthesis of Esters

Sym (66) developed a practical and excellent method for the enzymic synthesis of esters. He found that the sodium salts of bile acids, and sodium oleate respectively, which are able to form water-soluble addition compounds with fatty acids, cholesterol, aromatic hydrocarbons, etc., have an exceedingly great activating effect on enzymic ester synthesis. The synthesis, however, must be carried out in a medium of carbon tetrachloride or benzene, which are good solvents of the esters produced. The degree of esterification may be determined (*a*) by titrating the concentration of acid with 0.1 *N* sodium hydroxide; (*b*) by measuring the alcohol concentration, or (*c*) by measuring the ester formation. The esters may be readily isolated owing to their solubility in the organic solvent employed.

Preparation of Enzyme Material. Pancreas glands are finely ground in a meat chopper, twice extracted with five times their weight of acetone, and dried in the air. The dried tissue is chopped once more, and is sifted. The siftings contain 8 per cent of water, and are ready for use.

Synthesis of Butylbenzoate. To 25 cc. of carbon tetrachloride containing 0.45 *M* benzoic acid, and 0.5 *M* butyl alcohol, 2 grams pancreas powder, and 1 cc. of a 30 per cent solution of bile salts (sodium salts of bile acids prepared from ox bile) were added. The mixture was shaken for twenty-four hours in a water-thermostat of 37°. After twenty-four hours the concentration of the ester formed was 0.31 *M*, and after forty-eight hours it was 0.39 *M*. In a parallel experiment in which the bile salt solution was

TABLE VIII

CHOLINE ESTERASE AND ESTERASE ACTIVITIES OF VARIOUS BLOOD SERA

	Substrate	cc. 0.02 *N* NaOH required to titrate acid liberated in 20 minutes			Diminution in drop number in 40 minutes Tributyrin
		Butyryl-choline	Acetyl-choline	Methyl butyrate	
A	Monkey	8.8	3.45	0.15	13
	Man	4.95	2.6	0.15	10
	Dog	4.8	2.45	0.15	4
	Fox	2.4	....	0.05	0
B	Horse	5.45	2.0	2.95	24
	Guinea-pig	3.75	1.35	4.7	72
	Cat	2.15	....	0.55	36
	Cat	1.9	....	0.55	60
C	Pigeon	0.3	....	16.6	80
	Goose	0	....	3.65	44
	Duck	0.2	....	2.85	21
	Tortoise	0	....	1.95	..
	Mouse	0.4	....	1.75	96
D	Pig	0.6	....	0	0
	Fowl	0.5	....	0.25	4
	Fowl	0.7	....	0.3	3
E	Rat	0.05	....	1.05	18
	Rabbit	0.05	....	0.3	22
	Rabbit	0.1	....	0.25	23.5
F	Goat	0.1	....	0	1
	Ox	0.05	....	0.05	0
	Sheep	0.1	....	0.05	0
	Ferret	0	....	0.05	11
	Frog	0	....	0	0
	Hedgehog	0	....	0	0

substituted for 1 cc. of water, the ester formation was 16 times slower. Similar results may be obtained by using benzene instead of carbon tetrachloride.

Synthesis of Cholesterol Ester. To 12.5 cc. of carbon tetrachloride, containing 0.5 *M* of cholesterol, and 0.5 *M* of butyric acid, 1 gram of pancreas powder and 1 cc. of a 15 per cent bile salt solution were added. After twenty-four hours at 37° the concentration of ester formed was 0.4 *M*. No ester formation took place when 1 cc. of water was used instead of 1 cc. of bile salt solution.

Sym and associates have synthesized a number of esters by this method, and Rona, Ammon, and Fischgold (67) synthesized wax (cetylpalmitate) by it.

REFERENCES

1. Abderhalden, E., and Weil, A.: Studien über Lipasewirkung. *Fermentforschung*, **4**, 76 (1920–21).
2. Willstätter, R., and Memmen, F.: Über die Wirkung der Pankreaslipase auf verschiedene Substrate. *Z. physiol. Chem.*, **133**, 229 (1924).
3. Willstätter, R., and Waldschmidt-Leitz, E.: Über Pankreaslipase. Zweite Abhandlung über Pankreasenzyme. II. Leitlinein der Methode. *Z. physiol. Chem.*, **125**, 140 (1925).
4. Willstätter, R., and Waldschmidt-Leitz, E.: Über Pankreaslipase. *Z. physiol. Chem.*, **125**, 132 (1923).
5. Glick, D., and King, C. G.: The protein nature of enzymes. An investigation of pancreatic lipase. *J. Am. Chem. Soc.*, **55**, 2445 (1933).
6. Bamann, E., and Laeverenz, P.: Über pankreatische Lyo- und Desmolipasen. Vierte Abhandlung. Zur Kenntnis zellgebundener Enzyme der Gewebe und Drüsen in der von R. Willstätter und M. Rohdewald begonnenen Untersuchungsreihe. *Z. physiol. Chem.*, **223**, 1 (1934).
7. Rosenheim, O.: On pancreatic lipase. III. The separation of lipase from its co-enzyme. *J. Physiol.* **40**, XIV (1910).
8. Woodhouse, D. L.: Investigations in enzyme action directed towards the study of the biochemistry of cancer. The activation of pancreatic pro-lipase. *Biochem. J.*, **26**, 1512 (1932).
9. Falk, K. G.: Studies on enzyme action. V. The action of neutral salts on the activity of castor bean lipase, *J. Am. Chem. Soc.*, **35**, 601 (1913).

9*a*. Glick, D., and King, C. G.: Relationships between the activation of pancreatic lipase and the surface effects of the compounds involved. The mechanism of inhibition and activation. *J. Biol. Chem.*, **97**, 675 (1932).

10. Parfentjev, I. A., Devrient, W. C., and Sokoloff, B. F.: The influence of sodium taurocholate and copper sulfate on lipase. *J. Biol. Chem.*, **92**, 33 (1931).

11. Dakin, H. D.: The hydrolysis of optically inactive esters by means of enzymes. Part 1. The action of lipase upon esters of mandelic acid. The resolution of inactive mandelic acid. *J. Physiol.*, **30**, 253 (1903–1904).

11*a*. Dakin, H. D.: The fractional hydrolysis of optically inactive esters by lipase. Part II. *J. Physiol.*, **32**, 199 (1904–1905).

12. Willstätter, R., and Memmen, F.: Vergleich von Leberesterase mit Pankreaslipase; über die stereochemische Spezifität der Lipasen. *Z. physiol. Chem.*, **138**, 216 (1924).

13. Rona, P., and Ammon, R.: Die stereochemische Spezifität der Esterasen und die synthetisierende Wirkung der Ester-Spaltende Fermente. *Ergebnisse Enzymforschung*, **2**, 50 (1935).

14. Weinstein, S. S., and Wynne, A. M.: Studies on pancreatic lipase. I. *J. Biol. Chem.*, **112**, 641 (1936).

15. Willstätter, R., Waldschmidt-Leitz, E., and Memmen, F.: Bestimmung der pankreatischen Fettspaltung. (Erste Abhandlung über Pankreasenzyme.) *Z. physiol. Chem.*, **125**, 93 (1923).

16. Rona, P., and Michaelis, L.: Über Ester- und Feltspaltung im Blute und im Serum. *Biochem. Z.*, **31**, 345 (1911).

17. Rona, P.: Über Esterspaltung in den Geweben. *Biochem. Z.*, **32**, 482 (1911).

18. Davidsohn, H.: Beitrage zum Studium der Magenlipase. *Biochem. Z.*, **45**, 284 (1912).

18*a*. Davidsohn, H.: Über die Abhängigkeit der Lipase von der Wasserstoffionenkonzentration. *Biochem. Z.*, **49**, 249 (1913).

19. Glick, D.: Studies on enzymic histochemistry. VIII. A micromethod for the determination of lipolytic enzyme activity. *Compt. rend. trav. lab. Carlsberg*, **20**, No. 5 (1934).

20. Willstätter, R., and Memmen, F.: Vergleich von Magenlipase mit Pankreaslipase. *Z. physiol. Chem.*, **133**, 247 (1924).

21. State, W.: Untersuchungen über das fettspaltende Ferment des Magens. *Hofm. Beitr. Chem. Phys. Path.*, **3**, 291 (1903).

22. Kastle, J. H., and Loevenhart, A. S.: Concerning lipase, the fat-splitting enzyme, and the reversibility of its action. *Am. Chem. J.*, **24**, 491 (1900).

23. Kastle, J. H., Johnston, M. E., and Elvove, E.: Hydrolysis of ethyl butyrate by lipase. *Am. Chem. J.*, **31**, 521 (1904).

24. Sobotka, H., and Glick, D.: Lipolytic enzymes. II. The influence of hydrogen-ion concentration on activity of liver esterase. *J. Biol. Chem.*, **105**, 221 (1934).
25. Dakin, H. D.: The fractional hydrolysis of optically inactive esters by lipase. Part II. *J. Physiol.*, **32**, 199 (1904).
26. Pierce, G.: The partial purification of the esterase in pig's liver. *J. Biol. Chem.*, **16**, 1 (1913).
27. Kraut, H., and Rubenbauer, H.: Über Leberesterase. Versuche zu ihrer Reinigung und über ihre Beständigkeit. *Z. physiol. Chem.*, **173**, 103 (1928).
28. Baker, Z., and King, C. G.: The purification, specificity and inhibition of liver esterase. *J. Am. Chem. Soc.*, **57**, 358 (1935).
29. Willstätter, R., Kuhn, R., Lind, O., and Memmen, F.: Über Hemmung der Leberesterase durch Ketocarbonsäureester. *Z. physiol. Chem.*, **167**, 303 (1927).
30. Glick, D., and King, C. G.: Relationships between the activation of pancreatic lipase and the surface effects of the compounds involved. *J. Biol. Chem.*, **97**, 675 (1932).
30*a*. Sobotka, H., and Glick, D.: Lipolytic enzymes. I. Studies on the mechanism of lipolytic enzyme actions. *J. Biol. Chem.*, **105**, 199 (1934).
31. Green, J. R.: On the germination of the seed of the castor-oil plant (*Ricinus communis*). *Proc. Roy. Soc.*, **48**, 370 (1890).
32. Falk, K. G.: Studies on enzyme action. IX. Extraction experiments with the castor bean lipase. *J. Am. Chem. Soc.*, **35**, 1904 (1913).
33. Willstätter, R., and Waldschmidt-Leitz, E.: Über Ricinuslipase. *Z. physiol. Chem.*, **134**, 161 (1923–24).
34. Hoyer, E.: Über fermentative Fettspaltung. *Z. physiol. Chem.*, **50**, 414 (1906–07).
34*a*. Longenecker, H. E., and Haley, D. E.: Ricinus lipase, its nature and specificity. *J. Am. Chem. Soc.*, **57**, 2019 (1935).
35. Willstätter, R., and Stoll, A.: Untersuchungen über Chlorophyll. Berlin, 1913.
36. Willstätter, R., and Stoll, A.: Untersuchungen über Chlorophyll. *Ann.*, **378**, 18 (1911).
37. Willstätter, R., and Hug, E.: Isolierung des Chlorophylls. *Ann.*, **380**, 177 (1911).
38. Fernbach, A.: Chimie physiologique. Sur la tannase. *Compt. rend.*, **131**, 1214 (1900).
39. Pottevin, H.: Chimie physiologique. La tannase. Diastase deboublant l'acide gallotannique. *Compt. rend.*, **131**, 1215 (1900).
40. Freudenberg, K.: Die Chemie der natürlichen Gerbstoffen. Berlin, 1920; *Z. angew. Chem.*, **34**, 247 (1921).

41. **Dyckerhoff**, H., and **Armbruster**, R.: Zur Kenntnis der Tannase. *Z. physiol. Chem.*, **219**, 38 (1933).
42. **Rhind**, D., and **Smith**, F. E.: Note on tannase. *Biochem. J.*, **16**, 1 (1922).
43. **Neuberg**, C.: Über das neue Ferment Sulfatase. *Naturwissenschaften*, **12**, 797 (1924).
44. **Rosenfeld**, L.: Über das Vorkommen und Verhalten der Sulfatase in menschlichen Organen. *Biochem. Z.*, **157**, 434 (1925).
44*a*. **Tanko**, B.: Spaltung der Glucose-Schwefelsäure und Saccharose-Schwefelsäure durch Bakteriensulfatase. *Biochem. Z.*, **247**, 486 (1932).
44*b*. **Bodansky**, O.: The accelerant effect of α-amino acids on the activity of bone phosphatase. *J. Biol. Chem.*, **114**, 273 (1936); **115**, 101 (1936).
45. **Kay**, H. D.: Kidney phosphatase. *Biochem. J.*, **20**, 791 (1926).
46. **Waldschmidt-Leitz**, E., and **Köhler**, F.: Zur Specifität der Nierenphosphatase, *Biochem. Z.*, **258**, 360 (1933).
47. **Bamann**, E., and **Diedrichs**, K.: Trennung der beiden isodynamen Phospho-esterasen tierischer Organe durch ein selectives Inactivierungs-Verfahren (Dritte Abhandlung). Zur Kenntnis der Phosphatasen). *Ber.* (B), **67**, 2019 (1934).
48. **Akamatsu**, S.: Über das Vorkommen von Glycero-phosphatase in der "Takadiastase." *Biochem. Z.*, **142**, 184 (1923).
49. **Inouye**, K.: Die *p*H-Abhängigkeit der Glycerophosphatase. *J. Biochem. Tokyo*, **10**, 133 (1928).
50. **Roche**, J.: Blood phosphatases. *Biochem. J.*, **25**, 1724 (1931).
51. **Takahashi**, H.: Über Fermentative Dephosphorierung der Nukleinsäure. *J. Biochem. Tokyo*, **16**, 463 (1932).
52. **Folley**, S. J., and **Kay**, H. D.: The phosphatases. *Ergebnisse Enzymforschung*, **5**, 159 (1936).
53. **Bodansky**, A., and **Jaffe**, H. L.: Phosphatase studies. *Am. J. Diseases Children*, **48**, 1268 (1934).
54. **Roberts**, W. M.: Blood phosphatase and the Van den Bergh reaction in the differentiation of the several types of jaundice. *Brit. Med. J.*, **1**, 734 (1933).
54*a*. **Tenner**, H. D., and **Kay**, H. D.: Plasma phosphatase. *Brit. J. Exptl. Pathol.*, **13**, 22 (1932).
54*b*. **Schoonover**, J. W., and **Ely**, J. O.: Enzymes in cancer. The β-glycerophosphatase of the erythrocytes. *Biochem. J.*, **29**, 1809 (1935).
54*c*. **Bodansky**, A.: Phosphatase studies. II. Determination of serum phosphatase. *J. Biol. Chem.*, **101**, 93 (1933).
55. **Dale**, H. H.: The action of certain esters and ethers of choline, and

their relation to muscarine. *J. Pharm. Exptl. Therap.*, **6**, 147 (1914–15).

56. Hunt, R.: Note on a blood-pressure-lowering body in the suprarenal gland. *Am. J. Physiol.*, **3**, XVIII (1899–1900).

56*a*. Hunt, R.: Further observation on the blood-pressure-lowering bodies in extracts on the suprarenal gland. *Am. J. Physiol.*, **5**, vi (1901).

57. Hunt, R., and Renshaw, R. R.: Further studies of the methyl cholines and analogous compounds. *J. Pharm. Exptl. Therap.*, **51**, 237 (1934).

58. Dale, H. H.: Chemical ideas in medicine and biology. *Science*, **80**, 343 (1934).

59. Stedman, E., Stedman, E., and Easson, L. H.: Choline-esterase. An enzyme present in the blood-serum of the horse. *Biochem. J.*, **26**, 2056 (1932).

59*a*. Stedman, E., Stedman, E., and White, A. C.: A comparison of the choline-esterase activities of the blood-sera from various species. *Biochem. J.*, **27**, 1055 (1933).

60. Feldberg, W.: Neuere Versuche zur Physiologie des Acetylcholins. *Klin. Wochschr.*, **12**, 1036 (1933).

61. Govaerts, P., Cambier, P., and Von Dooren, F.: Vitesse de destruction de l'acetylcholine par le sang des individus sensibles ou résistants a cette substance. *Compt. rend. soc. biol.*, **108**, 1178 (1931).

62. Carmichael, E. A., and Fraser, F. R.: The effects of acetyl choline in man. *Heart*, **16**, 263 (1933).

63. Ammon, R., and Voss, G.: Die Acetylcholinzerstörende Wirkung des Blutes. *Pflügers Arch.*, **235**, 235 (1934–35).

64. Ammon, R.: Die Cholinesterase. *Ergebnisse Enzymforschung*, **4**, 102 (1935).

65. Plattner, F., and Hintner, H.: Die Spaltung von Acetylcholin durch Organextrakte und Körperflüssigkeiten. *Pflügers Arch.*, **225**, 19 (1930).

66. Sym, E. A.: Eine Methode der enzymatischen Estersynthese. *Enzymologia*, **1**, 156 (1936).

67. Rona, P., Ammon, R., and Fischgold, H.: Zur Kinetik der enzymatischen Esterbildung. *Biochem. Z.*, **241**, 460 (1931).

CHAPTER III

PROTEOLYTIC ENZYMES AND PEPTIDASES

THE STRUCTURE OF THE PROTEIN MOLECULE AND ITS RELATION TO PROTEOLYSIS

Facts in Support of a Polypeptide Structure

The view of a number of investigators is that all proteolytic enzymes split proteins by opening peptide linkages (—NH—CO—) in such a manner that equal numbers of amino and carboxyl groups are formed (1–10). This is in accordance with the classical theory of Emil Fischer. The structure of the substrate is an important factor in enzyme chemistry. According to Emil Fischer the amino acids are linked via the amino group of one amino acid and the carboxyl group of another, forming a long chain of a so-called polypeptide. Vickery and Osborne (11) tabulated the considerations which support Fischer's theory as follows:

1. Native protein itself contains very little amino nitrogen, but the end products of protein hydrolysis contain larger amounts. The peptide bond type of union readily accounts for this.
2. The biuret reaction is given by many substances which contain this group, and this reaction is characteristic of proteins and their decomposition products, the proteoses. It disappears on complete hydrolysis. This strongly suggests the presence of the peptide bond in proteins and their partial hydrolysis products.
3. A number of condensation products of amino acids have been prepared which contain this group. Many of these give the biuret reaction.
4. The peptide union is also encountered in other naturally occurring substances, as, for example, in hippuric acid.

5. The synthetic polypeptides obtained by Fischer from the natural isomers of optically active amino acids are hydrolyzed by the enzymes of the digestive tract.

6. Polypeptides have frequently been found among the products of incomplete hydrolysis of proteins.

7. During the hydrolysis of proteins, whether by acids or enzymes, amino groups and carboxyl groups are progressively liberated at an approximately equal rate.

8. Hydrolysis of proteins occurs without material change in the hydrogen-ion concentration of the solution. This is consistent with the view that equivalent amounts of amino and carboxyl groups are thereby produced.

9. Pepsin alone as a rule liberates about 20 per cent of the total amount of amino nitrogen which can be obtained by the complete hydrolysis of a protein. Erepsin, acting on a peptic digest, can liberate as much as 70 per cent more. Since there is every reason to believe that erepsin acts only upon peptide bonds, it is obvious that by far the greater part of the total possible amino nitrogen of a protein has its origin in such bonds.

Many facts, however, indicate that the protein molecule is not simply a long polypeptide. Polypeptides are not denatured by alcohol or heat. Polypeptides are water soluble; many proteins are not. Pepsin does not act on polypeptides.

It has been shown recently that the methods used for the estimation of the NH_2 and COOH groups, on which the peptide linkage theory is based, are not very specific (12, 13).

Abderhalden and associates (14–18) suggested years ago that proteins are built of a number of complexes containing diketopiperazins which are combined by latent valence (see also references 19 and 20).

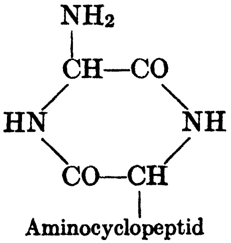
Facts in Support of a Diketopiperazin Structure

Abderhalden and Schwab (21) showed that trypsin and erepsin respectively can hydrolyze the following deketopiperazine derivatives: leucylglycyltyrosine anhydride, leucylglycylleucine anhydride, and leucylglycinserine anhydride. These compounds have recently been obtained in stable form for the first time.

Matsui (22) obtained similar results with asparagyldiglycyltyrosine, a synthetic tetrapeptide. The same worker (23) showed that synthetic diketopiperazinecarboxylic acid was split by trypsin, but not by pepsin or by erepsin. Ishiyama (24) studied the digestibility of glycylaspartic anhydride, glycylglutamic anhydride, aspartic anhydride, glycylglutamine anhydride, glycine anhydride, pyrrolidonecarboxylic acid, and pyrrolidonecarboxamide. The first three compounds were not split by erepsin or by pepsin, whereas trypsin rapidly hydrolyzed them. The remainder of the compounds were not attacked by the enzymes. A free carboxyl group is essential if the diketopiperazine molecule is to be split by trypsin; this was the case in the first three compounds. Amidation of the carboxyl group is sufficient to make these compounds indigestible; for example, glycylglutamine anhydride is very resistant.

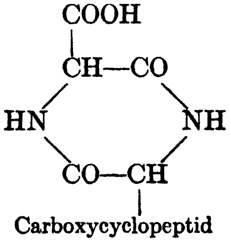
Theory of Shibata. According to Shibata (25), basic diketopiperazines like glycyldiaminopropionic anhydride and diaminopropionic anhydride are split by *pepsin* which is an " aminocyclopeptidase " (I), and acid diketopiperazines like glycylglutamic anhydride with a free COOH group and asparaginic anhydride are split by *trypsin, by papain, and by cathepsin, which are "carboxy-cyclopeptidases* (II):

(I)



Aminocyclopeptid

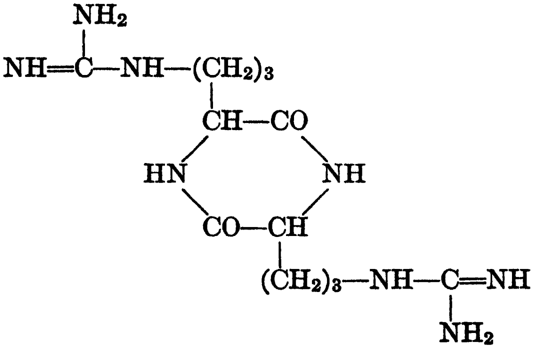
(II)



Carboxycyclopeptid

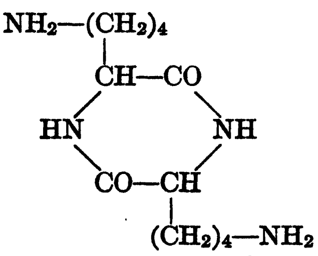
Tazawa (26) verified the theory of Shibata. He found that the anhydride (diketopiperazine) linkage of *d*-arginine anhydride (*A*) and *d*-lysin anhydride (*B*) was rapidly split by pepsin but not by trypsin or by papain. The dipeptides arginylarginine and lysyllysin, which were formed by the opening of the respective anhydrides by pepsin, were readily split by yeast dipeptidase. The optimum *p*H is at 2.2 to 2.4 for the pepsin action. Tazawa described also the synthesis of the two diketopiperazines (see also reference 27). These findings harmonize with Northrop's theory, which postulates that pepsin acts on cations and trypsin on anions of proteins (see Chapter I).

(*A*)



d-Arginine anhydride

(*B*)



d-Lysin anhydride

Thus it may be said that there are at least as many reasons to believe that the protein molecule is a diketopiperazine compound as there are for the assumption that it is a long polypeptide chain.

Svedberg's findings concerning the size of the protein molecule are interesting. He determined that aqueous solutions of protein contain definite particles, so-called macro molecules. Some proteins were found to have a molecular weight of 35,000, others of about 2,000,000 (28–34).

Goddard and Michaelis (35) found that the protein keratin which is not attacked by trypsin and pepsin because of the closely bound cystine linkages may be changed into a digestible form by a simple hydrogenation of the disulfide group. The reagent used is thioglycolic acid, which does not affect the peptide chains of keratin. The SH-keratin has movable peptide linkages and is readily digested. It is soluble in acid and alkali, with a definite isoelectric point.

MAMMAL GASTRIC PROTEASES

The protein-digesting property of gastric juice was noticed about 100 years ago by Schwann (36), who called the enzyme responsible for this effect, pepsin. Pepsin is secreted in great quantities by the chief cells of the gastric mucosa. It is the only protease of the mammal's (young and adult) stomach. An exception is the calf's fourth stomach which contains, in addition to rennin, some pepsinase. The protease of the adult and young mammal has both high protein-digesting power and high milk-clotting activity.

That small amounts of special proteolytic enzymes like gelatinase (37, 38) and kathepsin (39) are present in the gastric mucosa has been reported by various authors.

Tauber and Kleiner (40) have succeeded in separating rennin of the calf's fourth stomach from pepsin in so far as it had only negligible peptic activity and extremely high milk-coagulating power and different properties from pepsin.

The Rennin-Pepsin Problem

Propepsin or Pepsinogen and Prorennin

Pepsin or pepsinase, as formed by the gastric cells, is completely inactive. It cannot digest protein or clot milk

(41). This pre-stage is called precursor or zymogen. If the ground gastric mucosa of a pig is extracted with a suspension of $CaCO_3$ in water and some of the filtrate is added to milk, no clot will form. Adjustment of a sample of the $CaCO_3$ extract to about *p*H 2.0 converts all the propepsin to active pepsin which digests protein and also has a high milk-clotting power. A similar extract of the calf's fourth stomach shows considerable clotting power, even before acidification, and also becomes completely active when acid is added (41).

Preparation of Crystalline Pepsinogen

Pepsinogen has been isolated in crystalline form by Herriott and Northrop (41*a*) from alkaline, 0.45 saturated ammonium sulfate extracts of the swine fundus mucosae. The crystals (Fig. 7) were of protein nature. Pepsin prepared by the acidification of this pepsinogen can be crystallized, and its crystalline form is identical with pepsin crystallized from commercial pepsin. (The preparation of crystalline pepsin from commercial pepsin will be described elsewhere in this chapter.)

The following is the procedure for crystallizing pepsinogen:

(*a*) Minced swine fundus mucosae are extracted with one volume of 0.45 saturated ammonium sulfate in *M* 0.10 sodium bicarbonate equivalent to four times the weight of mucosae used and filtered after the addition of 10 per cent by weight Johns-Manville Filter Cel and 5 per cent by weight Hyflow Super Cel (Eimer and Amend).

(*b*) Pepsinogen is precipitated from 0.7 saturated ammonium sulfate, filtered, and dissolved in water.

(*c*) Pepsinogen is adsorbed from solution (*b*) at *p*H 6.0 by cupric hydroxide suspension and eluted in *M* 0.10 *p*H 6.8 phosphate.

(*d*) Treatment with cupric hydroxide is repeated.

(*e*) Soluble carbohydrate remaining is removed by treating with Filter Cel at *p*H 7.0.

(*f*) Pepsinogen crystallizes in fine needles over night at 10°, 0.4 to 0.45 saturated ammonium sulfate and *p*H 5.2 to 5.6 (orange red to methyl red).

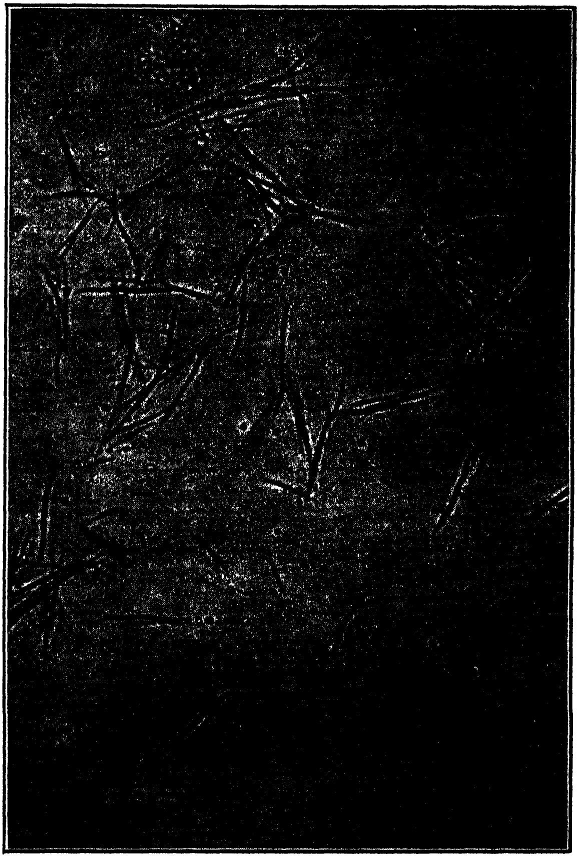


FIG. 7.—Crystalline pepsinogen

Chemical Differences between Rennin and Pepsin

The very powerful rennin preparation which has been obtained by Tauber and Kleiner (40) by isoelectric fractional precipitation had practically no power to digest egg white and 1 gram of it clotted 4,500,000 grams of milk of *p*H 6.2 at 37° in ten minutes. The vacuum-dried preparation differs from Northrop's crystalline bovine pepsin (see below) in many ways. It is very easily soluble in slightly acidified water (0.04 *N* HCl) and is not coagulated by heat. It gives different protein color tests. It dialyzes through membranes. Pepsin is reversibly inactivated by NaOH, as found by Northrop (42), and is not very resistant to acids. Rennin behaves in the opposite way in both respects. Rennin (Tauber and Kleiner) was found to have an isoelectric point of about 5.4. This is far above that of 2.75, estimated by Northrop (43) for pepsin. Rennin of the fourth stomach of the calf is readily digested and inactivated by pig pepsin.

The elementary composition and the above properties show that rennin is a thioprotease. Pepsin is an albumin.

Gastric Proteases are Kind-Specific

The author (44) has shown that the gastric protease differs with each species. From the gastric mucosa of the pig, rabbit, and calf's fourth stomach may be prepared selective chymoinhibitors which point to the " individuality " of the rabbit's and the pig's pepsinase and of rennin and also show that there is no rennin in the pig's and adult rabbit's gastric mucosa.

Preparation of Specific Gastric Chymoinhibitors

The term " chymoinhibitors " has been introduced by the author (44) for substances which inhibit the milk-coagulating power of proteases. To 75 grams of washed and ground gastric mucosa add 150 cc. of a 2 per cent $CaCO_3$ suspension in distilled water. Stir for eight minutes and filter. To some of the filtrate add an equal volume of 95 per cent of ethyl alcohol and mix. To some of the alcohol-treated preparation, add HCl until *p*H 1.0 is reached; the filtrate of the mixture is inactive. The filtrate of the inactive zymogen to which no HCl has been added, but an equal volume of alcohol, exerts selective inhibition. Thus, the inhibitor prepared from the pig gastric mucosa inhibits pepsin markedly, but rennin only slightly, whereas the inhibitor from the calf's fourth stomach inhibits rennin considerably, but pepsin only slightly. The inhibitor of the adult rabbit's stomach mucosa inhibits pepsin but not rennin (see Table IX). Blood serum, urease-protein, and acetone inhibit pepsin much more than rennin.

TABLE IX

Specific Chymoinhibitors from Gastric Mucosa of Various Animals

Experiment No.		Clot formation
1	1 cc. pepsin inhibitor (pig gastric mucosa)	None in 1080 minutes
2	1 cc. pepsin + 1 cc. 50% alcohol	2
3	1 “ “ + 1 “ pepsin inhibitor	6
4	1 “ rennin + 1 “ “ “	Negligible inhibition
5	1 “ “ inhibitor (calf 4th stomach)	No clot in 60 minutes
6	1 “ “ + 1 cc. 50% alcohol	3
7	1 “ “ + 1 “ rennin inhibitor	20
8	1 “ pepsin + 1 “ “ “	Negligible inhibition
9	Inhibitor from adult rabbit gastric mucosa inhibits only pepsin but not rennin.	
10	Inhibitor from chicken gastric mucosa inhibits neither rennin nor pepsin.	

In each of these experiments 10 cc. of milk of *p*H 6.3 was used. The temperature was 37°. The pepsin is the acidified neutral extract of the pig gastric mucosa, and the rennin is the acidified neutral extract of the gastric mucosa of the fourth stomach of the calf.

A comparison of the ratio of proleolytic power and milk-clotting activity of the young mammal's gastric juice as compared to that of the adult's has been the object of several investigations. Recently, Holter and Andersen (45) have taken up this problem again. They collaborated and extended the earlier findings of Hammarsten (46); i.e., the proteases of the gastric juice of various animals differ, and the difference is most pronounced between the calf's protease and that of the other mammals.

Northrop (47) has devised a method by which a crystalline pepsinase may be obtained from bovine gastric juice. The crystals had the same form, optical activity, and specific activity as the crystalline pepsinase which he obtained from pig gastric mucosa. The solubility of the two enzymes, however, was different, indicating that they are not the same proteins.

The peptic activity of the pepsinases parallels the milk-clotting power in the case of the adult mammal as well as in the young. No separation of the two activities is possible except in the case of the calf. The ratio of pepsin-rennin activity as expressed in units, however, differs in various mammals' gastric secretions. In calves the quotient was 0.13–0.26, in cows 1.6; in children 2.7, and in adults 2.5; in young dogs 11.5, and in grown dogs 12.5; in grown pigs 0.50. These results mean that the gastric enzymes do not vary in the young and adult human or dog as they do during various ages in the calf (45). The early conception of Hedin (48) that gastric proteases are kind-specific is now corroborated by the researches of Tauber and of Holter and Andersen.

Rennin is the only typical milk-clotting enzyme, and it exists only in the fourth stomach of the calf, associated with a small amount of pepsin, from which it is separable.

The Chemistry of Milk Clotting

According to Linderstroem-Lang (49, 50), native casein is a system of three components, one of which acts as a protective colloid for the other two. An attack by any proteolytic enzyme on the protective colloid, under proper conditions (presence of Ca ions or other earthy alkalis, *p*H, temperature), causes milk to clot, the coagulum being calcium paracaseinate. Experiments corroborating this theory have been furnished by Tauber (44).

All proteolytic enzymes can clot milk. Tauber and Kleiner (51) have found that trypsin also clots milk, but only within a certain limited range of concentration. If too diluted or too concentrated, no coagulation will occur. Concentrated trypsin solution, like that used in protein-digestion experiments, changes the casein molecule so rapidly beyond the paracasein stage that the milk will not clot, even after the subsequent addition of a very active rennin solution. These experiments have been verified and extended by Clifford (52). This proves that the milk-clotting power is a function of the trypsin molecule. Kleiner and Tauber also found that the velocity of milk coagulation in all three cases—rennin, pepsin, and trypsin—is proportional to the hydrogen-ion concentration, so that if the milk is adjusted to a low *p*H in order to depress the proteolytic activity of the concentrated trypsin, coagulation may occur (see Table X).

TABLE X

MILK-CLOTTING ACTIVITY OF RENNIN, PEPSIN, AND TRYPSIN AT VARIOUS *p*H

*p*H	Clotting time of rennin	Clotting time of pepsin	Clotting time of trypsin
	minutes	minutes	minutes
5.6	3.5	1.5	3.0
5.9	4.5	2.0	4.0
6.1	8.0	3.0	6.0
6.4	10.0	11.0	No clot in 40 minutes*
6.6	29.0 very slight	No clot in 40 minutes	" " " 40 " *
6.8	36.0 " "	" " " 40 "	" " " 40 " *

The *p*H of the milk was adjusted with HCl or NaOH and electrometrically controlled. To 10-cc. samples of milk, 0.5 cc. of each of the enzyme solutions was added. The trypsin solution was 1 per cent. The pepsin solution contained 18,000 rennet units per cc. The rennin solution had an activity of 16,000 units per cc. Temperature 40°.

---

* No clot occurred after adjustment to *p*H 5.6.

Estimation of Rennet Activity

The velocity of the milk-clotting power increases as the milk is made more acid, until the isoelectric point of casein is reached and the casein precipitates without the addition of enzyme. A milk of *p*H 5.0 is most practical. Since the *p*H of cow's milk varies, the addition of an equal volume of *M* acetate buffer of *p*H 5 furnishes an excellent substrate (of *p*H 5) for the measurement of rennet activity. In each of a series of test tubes, 10 cc. of this milk (room temperature) is placed and also varying amounts of the enzyme solution to be tested. The test tubes are then corked and placed in a flat box and shaken at room temperature. The amount of enzyme which clots 10 cc. of the buffered milk in ten minutes at 18° is determined. A greater delay of the clotting time will yield results not proportional to the enzyme used. The use of a strongly buffered milk has been first suggested by Ege and Menck-Tygesen.

CRYSTALLINE PEPSIN

The adsorption method was not successful in the purification of either pepsin or rennin. Rennin, as shown, was obtained quite pure and of very high activity, by isoelectric fractional precipitation.

Northrop (53) noticed that the precipitate which formed in the dialyzing bag when the procedure for pepsin purification, of Pekelharing (54), was followed (Pekelharing's pepsin was relatively pure and very active) appeared in granular form and filtered readily, as if it were on the verge of crystallization. This precipitate dissolved at 45°, and when filtered, it crystallized on cooling.

Northrop then devised a method by which crystalline pepsin may be obtained in large quantities:

Preparation of Crystalline Pepsin

Five hundred grams of Parke, Davis pepsin U.S.P. 1 : 10,000 is dissolved in 500 cc. H_2O, and 500 cc. *N* H_2SO_4 is added. To this, 1000 cc. saturated $MgSO_4$ is added with stirring. The solution is filtered through fluted paper and then filtered with suction. The filtrate is discarded. The

remaining precipitate is stirred with H_2O to a thick paste, and $M/2$ NaOH added to form a complete solution. Local excess of NaOH must be avoided, and the *p*H should never be more than 5.0. $M/2$ H_2SO_4 is now added with stirring until a heavy precipitate forms (*p*H about 3). Allow to stand from three to six hours at 8°; filter with suction. Discard filtrate. Stir precipitate to a thick paste at 45°. Add carefully $M/2$ NaOH until precipitate dissolves. Filter if necessary and discard precipitate. Place beaker in a vessel containing 4 liters of H_2O at 45° C. Inoculate and allow to cool slowly. This should require from three to four hours. A heavy crystalline precipitate forms at about 30 to 35°. Keep solution at 20° for twenty-four hours to complete crystallization. Filter with suction and wash with small amounts of cold H_2O and then with $MgSO_4$ at 5°. Figure 8 shows crystalline pepsin.

Recrystallization

Filter the crystalline paste with suction and wash three times with cold $M/500$ HCl. Stir filter cake to a paste with half its weight of water at 45° and add $M/2$ NaOH, constantly stirring until the solution is faintly turbid. Add a few crystals and allow the solution to cool slowly, as before. In twenty-four hours a heavy crop of crystals separates. The suspension is then warmed to 45° and more H_2SO_4 added until *p*H 3.0. Allow to cool slowly and filter after twenty-four hours. The crystals may be washed with $M/500$ HCl until free of SO_4.

Chemical Nature of Crystalline Pepsin

It has been shown by Northrop that crystalline pepsin is a single chemical substance. By repeated crystallization, the optical activity, the composition, as well as enzymic activity, remain unchanged. The solubility of the enzyme was tested in a number of salt solutions and the substance reacted as a single compound. Northrop also found that, if pepsin is partly denatured by heat at various pH's, the inactive enzyme was parallel to the denatured protein. Percentage activity left after heat inactivation is the same

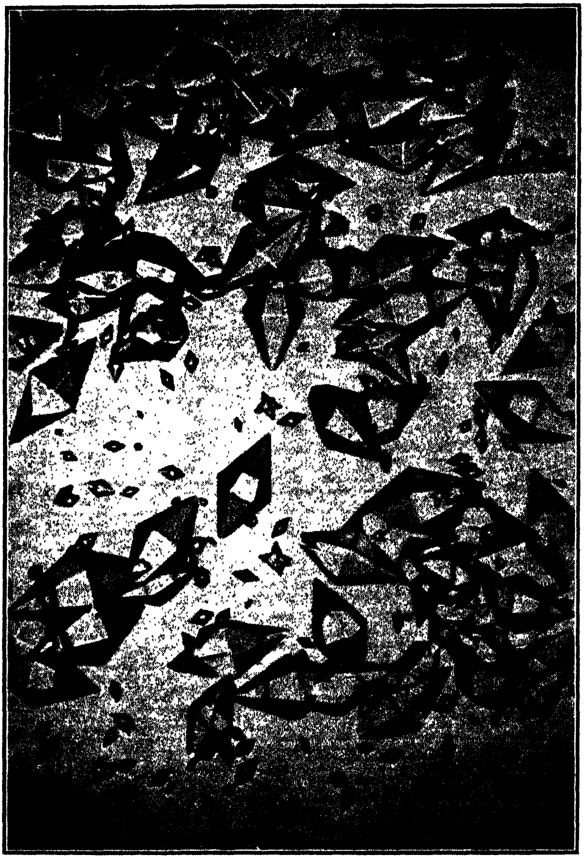


FIG. 8.—Crystalline pepsin

as percentage of soluble nitrogen remaining (see Fig. 9). Physical and chemical properties show that pepsin is an albumin. (See also " Chemical Differences between Pepsin and Rennin.")

Crystalline Acetyl Derivatives of Pepsin

Herriott and Northrop (55) acetylated crystalline pepsin with ketene in aqueous solution at *p*H 4.0–5.5. Three acetyl products have been isolated from the reaction mixture and prepared in crystalline form. These derivatives crystallize in the same form as the original pepsin, and a compound with three or four acetyl groups, which lost all its primary amino groups, was obtained on short acetylation. It had the same activity as the original pepsin. They obtained a second derivative with six to eleven acetyl groups, and this had an activity as great as that about 60 per cent of the original pepsin. A third derivative with twenty to thirty acetyl groups had also been obtained by prolonged acetylation. It was only about 10 per cent as active as the original pepsin.

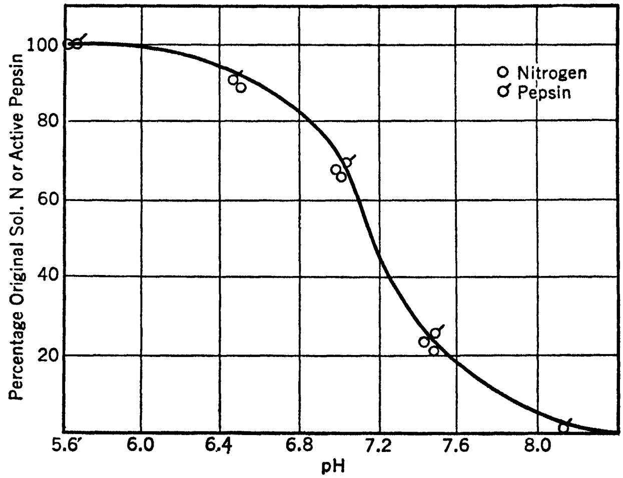


Fig. 9.—Percentage inactivation and percentage denaturization of Crystalline pepsin at various *p*H values at 20° C.

The authors furnish evidence which shows that the acetylation of three or four of the primary amino groups of pepsin effects no change in the activity of the enzyme, but the introduction of acetyl groups in other parts of the molecule results in a marked inactivation. Certain properties of the acetyl derivatives are different from those of the original crystalline pepsin; others, however, are not.

Optimum *p*H of Pepsin with Various Substrates

It has been known for many years that peptic digestion requires acid, whereas tryptic digestion may occur in a neutral or alkaline medium. Sörensen (56) was the first who pointed to the relationship between enzyme activity and hydrogen-ion concentration, and it was he who constructed the first activity *p*H curve. Michaelis (57) found that such curves could be calculated if it is assumed that the enzyme is a weak acid or base, and that the activity of the enzyme is a property of the ion or the undissociated molecule, according to the nature of the enzyme.

There is, however, no direct evidence for the ionic character of the enzymes. Migration experiments on pure pepsin contradict some of the assumptions used in the calculations (58). It was suggested that the acid affects the substrate and not the enzyme (59, 60). Northrop (61) showed experimentally that, in the case of pepsin and trypsin, the effect is primarily on the protein. He compared the effect of the addition of acid on the quantity of ionized protein with the effect on the rate of digestion of gelatin, casein, and hemoglobin by pepsin or trypsin. The rate of digestion may be predicted from the quantity of protein ion present. This was determined by the titration curve and conductivity. Pepsin attacks the acid salt of these proteins very rapidly, whereas trypsin attacks the alkali salt. The dissociation curve for proteins, therefore, is practically identical with the curve for the rate of digestion plotted as a function of the *p*H. Identical results have been obtained by Northrop with trypsin, edestin, and globin. The alkaline dissociation curve of these proteins harmonizes with the curve for the rate of trypsin digestion. The rate of digestion in the case of pepsin and trypsin is at a minimum at the isoelectric point of the protein and at a maximum at that hydrogen-ion concentration at which the protein is completely combined, forming a salt with acid or alkali. (See Table XI for optimum *p*H of trypsins.) Northrop (61) showed that the optimum *p*H of pepsin, with casein as a substrate, is 1.8, and with gelatin and hemoglobin, it is 2.2 for each. It should be noted that the hydrogen or hydroxyl ion is the determining factor. The nature or valence of the anion is of very little importance. Thus, peptic digestion proceeds equally well in a medium of hydrochloric acid, nitric acid, lactic acid, oxalic acid, or tartaric acid (Sörensen, Michaelis, Northrop).

Methods

Sörensen's formal titration method, by which the increase in amino groups may be determined in the presence of carboxyl groups, has been applied to the determination of peptic activity and proteolytic activity in general (62–65). Willstätter, Waldschmidt-Leitz, Dunaiturria, and Künstner (66) titrate COOH groups with NaOH in alcoholic solution in the presence of NH_2 groups. Van Slyke's (67) manometric method for the determination of amino nitrogen is often used. For a criticism of these methods see references 12 and 13.

Northrop (68) has given an excellent description of the following methods for the estimation of proteolytic activity: the Kjeldahl method for non-protein nitrogen; the estimation of protein nitrogen; the change in viscosity of protein solutions; milk clotting; and a modification of the formol method.

Anson and Mirsky (69) developed a convenient colorimetric method, using hemoglobin as a substrate and tyrosine as a standard.

TRYPTASES

Extracts of fresh pancreas or freshly secreted pancreatic juice have no proteolytic activity (70–72). The preparations become active when mixed with the enterokinase of the small intestine or when the pancreas is kept in a medium of slight acidity. The activation mechanism was a subject of controversy for several years, and the belief was that the mechanism of activation is a catalytic one; i.e., enterokinase is an enzyme. Vernon (73, 74) stated that the zymogen can be activated by trypsin as well as by enterokinase, but Bayliss and Starling (75) contradicted this. Later it was stated (Hamburger and Hekma, Dastre and Stassano, and Waldschmidt-Leitz [76]) that the reaction of activation is stoichiometric. The enterokinase forms an addition compound with the inactive zymogen. That at least two proteases are present in the pancreas, one which is not very stable and another of higher stability, and that the former can activate the latter, was the opinion of Vernon as early as 1902 (77).

Willstätter and Waldschmidt-Leitz (78) separated the pancreatic enzymes, " amylase, lipase and trypsin," by adsorption and elution. For a discussion of these methods, see reference 79. The separation was based on the different acidic and basic properties of these enzymes; i.e., acid or basic adsorbents such as various gels of alumina and kaolin were employed. Waldschmidt-Leitz and Harteneck (80) separated " erepsin," the peptide-hydrolyzing mixture, from the " proteinase " trypsin.

The product which Willstätter and Waldschmidt-Leitz obtained by the adsorption-elution method was identified by them as a mixture of " inactive trypsin " and a small percentage of " trypsin-enterokinase." A separation of the two has been reported by Waldschmidt-Leitz, Schäffner and Grassmann (81) and by Waldschmidt-Leitz and Linderstroem-Lang (82). Recently, Abderhalden and Schwab (83) reported on a further separation of the pancreatic proteases (see also reference 84).

Enterokinase

In 1924 Waldschmidt-Leitz (76) found that the activation of trypsin by enterokinase of the small intestine is a stoichiometric combination of inactive enzyme and activator. He claims to have separated spontaneously activated pancreatic trypsin by an adsorption method into inactive trypsin and activator. This fact speaks against an enzymic activation of trypsinogen. Contrary to this, however, enterokinase cannot be obtained by adsorption from the active trypsin-complex of leucocytes (85, 86). Waldschmidt-Leitz and Akabori (87) believe that pancreatic proteinase is a mixture of two trypsins (see below). It has been shown that the pancreas contains an activator which is identical with intestinal enterokinase (88).

In the pancreatic juice trypsinogen is accompanied by prokinase, which does not become active within the gland. It is activated in the cells of the intestinal mucosa. The intestinal juice does not contain any enterokinase, and its appearance here depends upon the pancreatic juice. After pancreatectomy, no appreciable amount of enterokinase is found in the intestinal mucosa (89).

Waldschmidt-Leitz (88) succeeded in concentrating enterokinase from the intestinal mucosa one hundred times. He found that the kinase is destroyed at 50°.

Pace (90) has described a method for the isolation of the precursor of enterokinase. This precursor, however, could not be separated from dipeptidase. The prokinase becomes active on standing. Pace believes that dipeptidase activates the prokinase. Bates and Koch (91) showed that an aqueous extract of fresh hog pancreas becomes completely active if kept at room temperature for twenty-four hours at *p*H 4 to 5. These authors have obtained trypsin-free trypsinogen by keeping the finely ground hog pancreas suspended in H_2O at *p*H 1.8 for three hours at 37° and adjusting the filtrate with NaOH to *p*H 6.0. A slight precipitate which formed was discarded, as was the one which formed on 45 per cent acetone concentration. The precipitate on 75 per cent acetone concentration contained the final trypsinogen. Bates and Koch also developed a method for enterokinase preparation, and demonstrate that enterokinase acts as an enzyme in the activation of trypsinogen. Their activation experiments, however, do not furnish evidence for an autocatalytic activation of trypsinogen.

Northrop (92), too, doubts the existence of the "trypsin-kinase-complex," and Waldschmidt-Leitz seems to have abandoned his earlier conception of its stoichoimetric formation (87).

From the above it appears that the rôle of enterokinase and its chemical nature deserve further investigation.

Methods for the estimation of enterokinase have been devised by Linderstroem-Lang and Steenberg (93) and by Bates (94).

Desmo- and Lyotrypsin

According to Willstätter and Rohdewald (95), dried pancreas, extracted several times with water-free glycerol, leaves about 30 per cent trypsin bound to the tissue. They call this " desmotrypsin," and the small amount of peptidase accompanying it, " desmopeptidase."

Desmotrypsin is inactive and may be activated by enterokinase. The kinase is also present in the form of an inactive precursor. If the pancreas is extracted slowly with aqueous glycerol, a spontaneous activation takes place which is complete in several weeks. Desmotrypsin may be fractionated into an α-fraction, which is soluble in the presence of electrolytes, and a β-fraction, soluble only in diluted sodium carbonate or HCl. The soluble or " lyotrypsin " carries much protein ballast, likewise the desmotrypsin. The latter, however, if subjected to " autotryptic " activation, yields a purer lyo-trypsin which gives only slight protein test. These tryptases are not claimed to be pure.

Crystalline Trypsin

Northrop and Kunitz (96, 97) described the preparation of a crystalline protein from frozen beef pancreas which had been allowed to thaw over night (spontaneous activation). It had a high tryptic activity, which remained constant, as did the optical activity under various conditions. The loss in activity corresponded to the decrease in native protein when the protein was denatured by heat, digested by pepsin, or hydrolyzed in diluted alkali. This enzyme, which Northrop and Kunitz named " trypsin," does digest casein, gelatin, edestin, peptone, and denatured hemoglobin, but is inactive to native hemoglobin. Peptides which were readily hydrolyzed by the original extract were not changed by the crystalline enzyme. Apparently the peptidases have been separated or rendered inactive by the procedure. This trypsin clots blood and milk. Experiments of Tauber and Kleiner have shown that trypsin can clot milk only under certain limited conditions (51). Enterokinase does not increase the activity of crystalline trypsin. It has an isoelectric point between *p*H 7 and 8, and optimum *p*H of casein digestion from 8 to 9. The stability is optimum at 1.8; that of crude trypsin is *p*H 6.5 (98).

Since crystalline trypsin follows a different course from crude material, Northrop and Kunitz advise against the method used by Willstätter and his associate for the determination of tryptic activity. There are possibly some activators or coenzymes in crude preparations which carry the hydrolysis further.

The preparation of crystalline trypsin by the direct method (96) is extremely difficult. It is much easier to prepare first crystalline trypsinogen from which crystalline trypsin may be obtained more readily (see below) than by the direct method.

The Effect of the Substrate Concentration on Tryptic Hydrolysis

The digestion of gelatin, casein (Fig. 10), and hemoglobin (Fig. 11) with crude and crystalline trypsin has been followed with varying amounts of the substrates at 35° C.

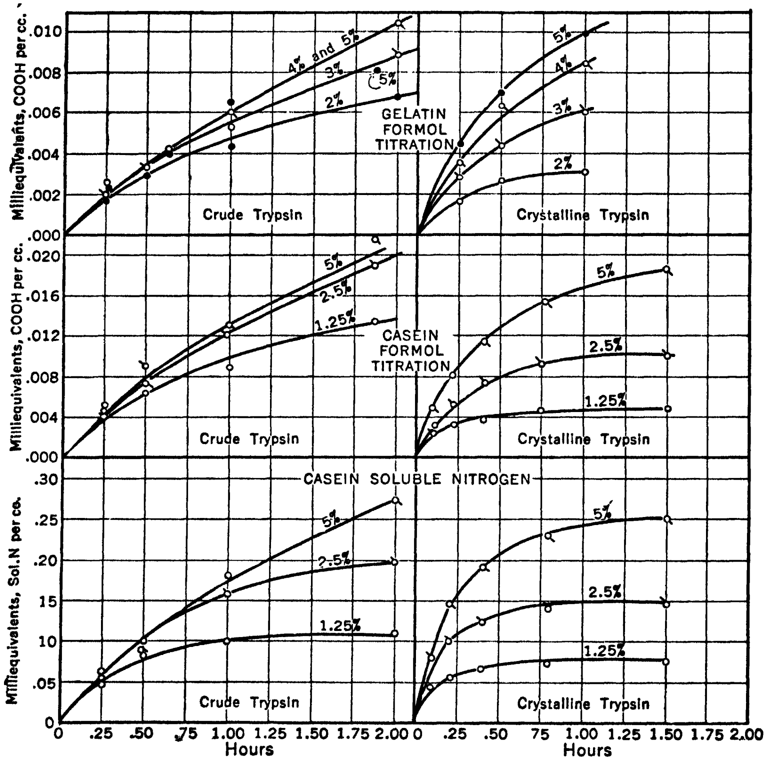


Fig. 10.—Digestion of various concentrations of gelatin and casein with crude and crystalline trypsin

For about 35 per cent of the reaction, the amount of digestion with crude trypsin is the same for 2.5 and 5 per cent protein concentration. The amount of digestion, instead of being proportional to the substrate concentration, becomes independent of it. This of course occurs often with enzymes and may be due to the formation of an intermediate compound. With crystalline trypsin, however, this anomaly is much less marked and the result is nearly that expected from the substrate concentration.

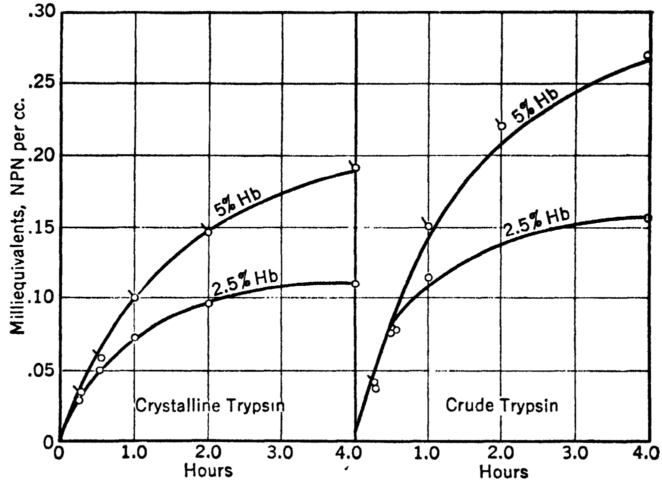


FIG. 11.—Digestion of hemoglobin with crude and crystalline trypsin

Crystalline Chymotrypsinogen and Crystalline Chymotrypsin

Kunitz and Northrop (99) attempted to isolate the inactive precursor of crystalline trypsin from fresh inactive (cattle) pancreatic extracts. They obtained a crystalline inactive protein and named it " chymotrypsinogen." *Enterokinase does not activate it but crude or crystalline trypsin changes* it into an active enzyme which they called " *chymotrypsin.*" This enzyme was also crystallized. It differs from chymotrypsinogen in the form of its crystals, the optical activity, and the number of amino groups. It is less stable and more soluble. The molecular weight is the same as that of chymotrypsinogen. This new enzyme does not clot blood like crystalline trypsin and has a weaker effect on protamins. Like crystalline trypsin, it attacks proteins in weak alkaline solution.

It has been demonstrated by Waldschmidt-Leitz and Akabori (87) that pancreatic proteinase (the trypsin of Waldschmidt-Leitz and associates) is probably a mixture of trypsin and chymotrypsin. These findings seem to confirm Vernon's experiments, which showed that in activated pancreatic extract there are at least two proteinases. The experiments of Kunitz and Northrop indicate that there are at least two inactive zymogens, chymotrypsinogen and trypsinogen, in fresh pancreatic extracts. Enterokinase changes trypsinogen to trypsin, and the trypsin transforms chymotrypsinogen into chymotrypsin. The formation of chymotrypsin from chymotrypsinogen is followed by a change in optical activity and increase in amino nitrogen. There is no change in the non-protein nitrogen fraction formed and in the molecular weight. Kunitz and Northrop believe that the activation may be caused by an internal molecular rearrangement.

According to these investigators, neither of these two crystalline substances changes its properties after repeated recrystallization. Tests such as denaturation and hydrolysis indicate that the enzyme and its precursor are pure proteins. Di- and polypeptides are not affected.

Preparation of Crystalline Chymotrypsinogen. Ten cattle pancreases are immersed in cold 0.25 N H_2SO_4 immediately after removal. The pancreas is freed from fat and connective tissue and minced in a meat grinder. Two volumes of ice-cold 0.25 N H_2SO_4 is added and the suspension is allowed to stand at 5° C. over night. It is then strained through gauze on a Buchner funnel and the precipitate suspended again in 0.25 N H_2SO_4 and refiltered. The combined filtrates are brought to 0.4 saturation with solid ammonium sulfate and filtered through a soft fluted filter at a low temperature. To the filtrate ammonium sulfate is added to 0.7 saturation and the suspension allowed to settle in the cold for forty-eight hours. The supernatant fluid is decanted and the suspension filtered with suction. The filter cake is dissolved in 3 volumes of

H_2O and 2 volumes of saturated ammonium sulfate added. The volume of the semi-dry cake is determined by weight and the specific volume is assumed to be equal to one. The suspension is filtered and the precipitate discarded. The filtrate is brought to 0.7 saturation with solid ammonium sulfate. The suspension is filtered with suction. The filter cake is dissolved in 1.5 volumes of H_2O and brought to $\frac{1}{4}$ saturated ammonium sulfate by the addition of saturated ammonium sulfate solution. The solution is adjusted to *p*H 5.0 (brick-red color with methyl red on test plate) with 5 *N* NaOH. About 1.5 cc. per 100 cc. is necessary. The solution is kept at 20° for two days. A heavy crop of crystals gradually forms. They are filtered with suction.

Recrystallization. The crystalline filter cake is suspended in 3 volumes of water and 5 *N* H_2SO_4 is added with stirring until the precipitate is dissolved. The solution is brought to $\frac{1}{4}$ saturated ammonium sulfate by the addition of 1 volume of saturated ammonium sulfate. Five *N* NaOH is added, with stirring, until the solution reaches *p*H 5, and kept at 20°. In an hour, crystallization should be completed. Yield: 15 grams of crystallized filter cake.

For the preparation of active chymotrypsin the crystallization should be repeated eight times as it would otherwise be difficult to obtain the active enzyme in crystalline form.

It has been shown by Kunitz and Northrop that the properties of the crystalline chymotrypsinogen are constant through ten fractional recrystallizations. Figure 12 shows crystals of chymotrypsinogen.

Activation of Chymotrypsinogen. The activity of recrystallized chymotrypsinogen is extremely slight and is only about 1/10,000 of chymotrypsin. It cannot be activated by enterokinase, calcium chloride, pepsin, inactivated trypsin, or chymotrypsin. It can be activated by commercial trypsin preparations and by crude active pancreatic extracts. The spontaneous activation is only 1 per cent in 1 month at 5°. The active trypsin-nitrogen used for the activation should be about 1/200 of the chymotrypsinogen-nitrogen, and the pH should be between 8 and 9.

Isolation and Crystallization of Chymotrypsin. Ten grams of eight times recrystallized chymotrypsinogen filter cake is suspended in 30 cc. H_2O and dissolved by the addition of a few drops of 5 *N* H_2SO_4; 10 cc. *M*/2, pH 7.6, phosphate buffer is added, and a quantity of *M* NaOH equivalent to the acid is added. About 0.5 mg. crystalline trypsin is added, and the solution is left at about 5° for forty-eight

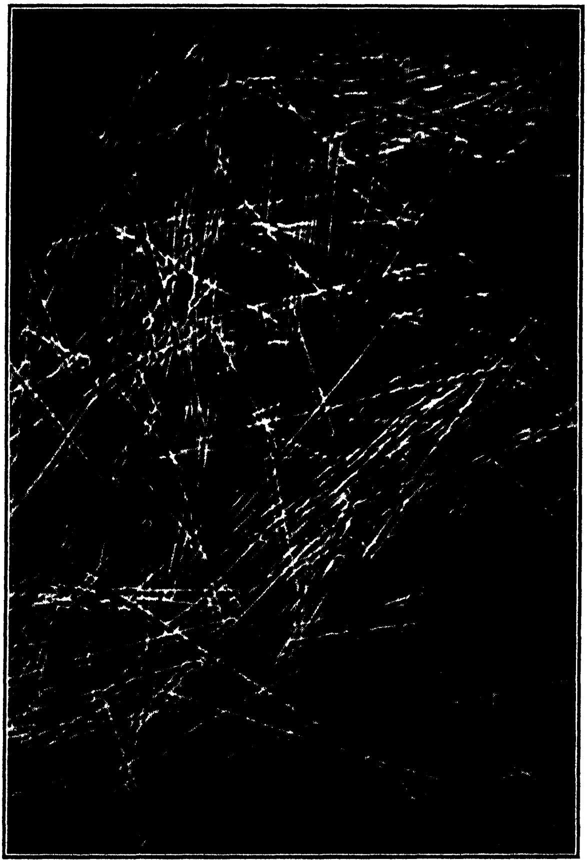


FIG. 12.—Chrystalline chymotrypsinogen

hours. The equivalent of any active trypsin may be used instead of the crystalline trypsin. After forty-eight hours the solution is brought to *p*H 4.0 by the addition of about 5 cc. $N/1H_2SO_4$. Twenty-five grams of solid ammonium sulfate is added, and the precipitate is filtered with suction. The filter cake is dissolved in 0.75 volume of $N/100$ H_2SO_4 and filtered if necessary. The clear filtrate is inoculated and allowed to stand at 20° for twenty-four hours. About 5 grams of crystalline filter cake should be obtained.

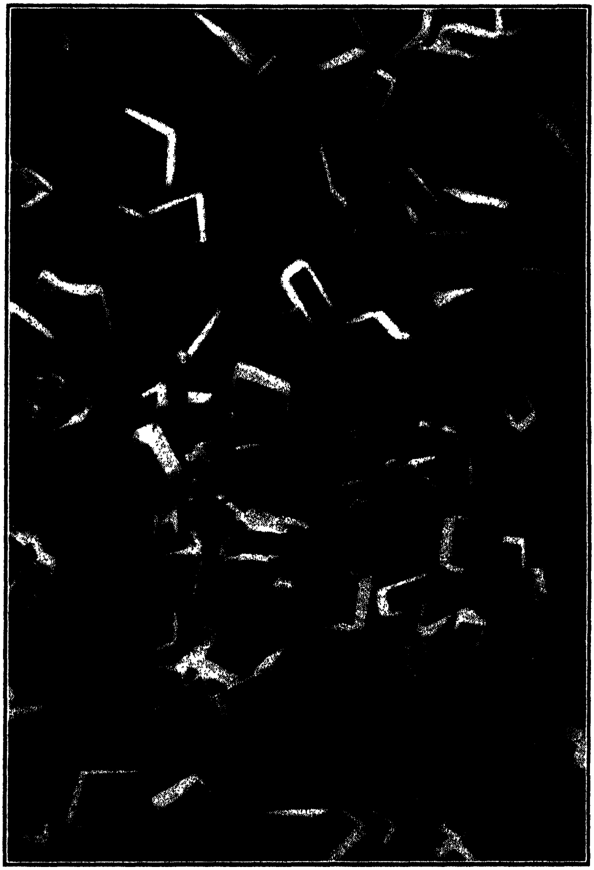


FIG. 13.—Crystalline chymotrypsin

Recrystallization. The crystalline filter cake is dissolved in 1.5 volumes $N/100$ H_2SO_4; 1 volume of saturated ammonium sulfate is then added carefully until crystallization commences. If the solution is allowed to stand at room temperature, complete crystallization should take place. The properties of the enzyme remain constant through three fractional crystallizations. Figure 13 shows crystals of chymotrypsin.

Crystalline Trypsinogen

Kunitz and Northrop (100) developed a method for the preparation of crystalline trypsinogen from the mother liquor from chymotrypsinogen. Magnesium sulfate at *p*H 7 to 8 activates this zymogen. The active trypsin can also be crystallized and is then identical with crystalline trypsin which has been described above. The following is the procedure for the preparation of crystalline trypsinogen: All the solutions must be cooled to about 5°, and all operations must be carried out in the ice box. The mother liquid from the chymotrypsinogen crystallization is adjusted to *p*H 4.0 with 2.5 *M* sulfuric acid, brought to 0.7 saturated ammonium sulfate, and filtered. One hundred grams of the precipitate is dissolved in 300 cc. water, brought to 0.4 saturated ammonium sulfate, and filtered. The filtrate is brought to 0.6 saturated ammonium sulfate by slow addition of saturated ammonium sulfate and filtered with suction. The precipitate is washed twice with saturated magnesium sulfate. Ten grams of filter cake is dissolved in 10 cc. 0.4 *M* borate buffer of *p*H 9.0; 17 cc. saturated magnesium sulfate is added, and the solution is allowed to stand at 6°. Short triangular pyramids form in the course of two to three days. Inoculation of the solution hastens crystallization. Sometimes the solutions become active and crystallization stops or crystals of the active trypsin may appear. Figure 14 shows crystalline trypsinogen, and Fig. 15 crystalline trypsin as obtained therefrom.

Conversion of Trypsinogen to Trypsin and Crystallization of Trypsin

The trypsinogen crystals are washed with 0.5 saturated magnesium sulfate in 0.10 *M* borate buffer of *p*H 8.0 and then with saturated magnesium sulfate in 0.1 *M* acetic acid. Ten grams of filter cake is suspended in 5 cc. 0.01 *M* sulfuric acid and 2.5 *M* sulfuric acid added drop by drop until the crystals dissolve. Ten cubic centimeters of saturated mag-

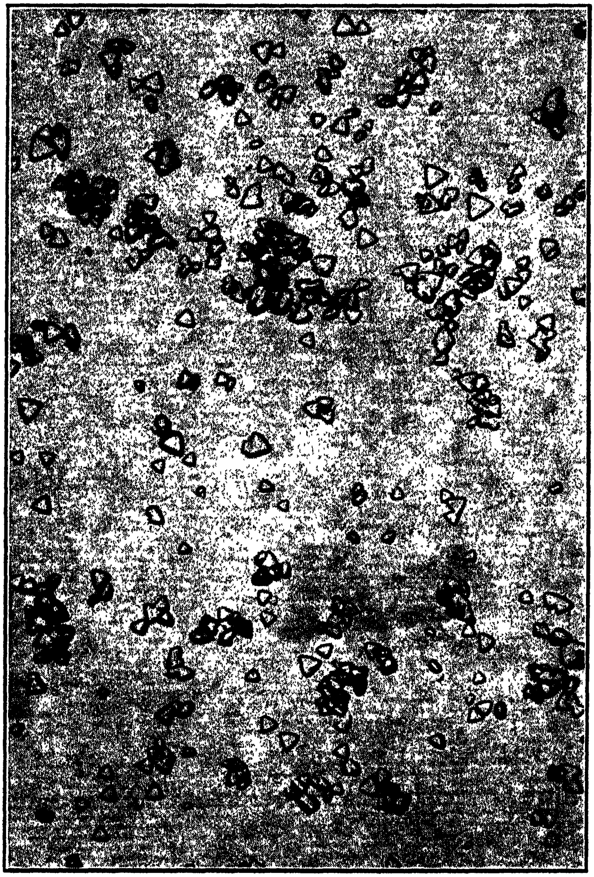


FIG. 14.—Crystalline trypsinogen

nesium sulfate and 5 cc. 0.4 *M* borate buffer of *p*H 9.0 are added, and the *p*H is adjusted with saturated potassium bicarbonate solution to pink to phenol red on a test plate. The solution is inoculated and kept at about 5°. A heavy crop of trypsin crystals forms in a few hours. Recrystallization is carried out in the same manner but with slightly more dilute solution. These crystals are needle-shaped and may be short or in rosettes. The purified trypsin obtained by the same investigators from active cattle pancreas, and

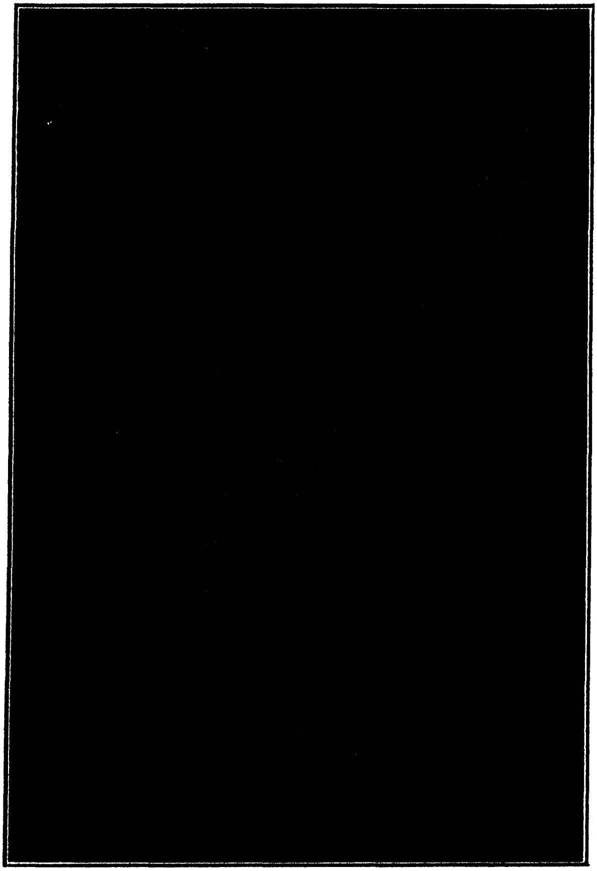


FIG. 15.—Crystalline trypsin

described above, may be crystallized by this method. The crystals are much better than those obtained at *p*H 4.0 and room temperature with ammonium sulfate.

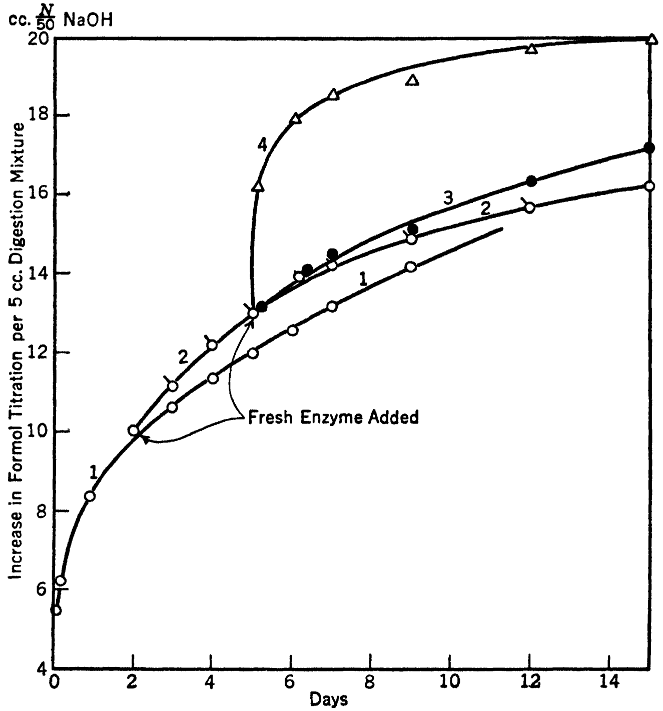


FIG. 16.—Digestion of casein by chymotrypsin followed by trypsin

100 cc. $\begin{cases} \text{5 per cent casein } p\text{H 7.6 (M/10 phosphate buffer)} \\ \text{0.08 mg. chymotrypsin nitrogen/cc} \end{cases}$ .... = No. 1

After 2 days 0.08 mg. chymotrypsin nitrogen/cc. added to 75 cc. No. 1 (Total chymotrypsin concentration 0.16 mg. nitrogen/cc.)...... = No. 2

After 5 days 0.08 mg. chymotrypsin nitrogen/cc. added to 25 cc. No. 2 (Total chymotrypsin concentration 0.24 mg. nitrogen/cc.)...... = No. 3

After 5 days 0.08 mg. crystalline trypsin nitrogen/cc. added to 25 cc. No. 2. (Total enzyme concentration 0.16 mg. chymotrypsin nitrogen/cc. plus 0.08 mg. trypsin nitrogen/cc.).............. = No. 4

Digestion determined by formol titration.

For further details on the preparation of the crystalline trypsins, see reference 100*a*.

Extent of Hydrolysis of Casein by Chymotrypsin

Casein is split more completely by chymotrypsin than by crystalline trypsin (96). Hydrolysis by the two enzymes, however, takes place at different linkages. This is indicated by the fact that addition of trypsin to casein previ-

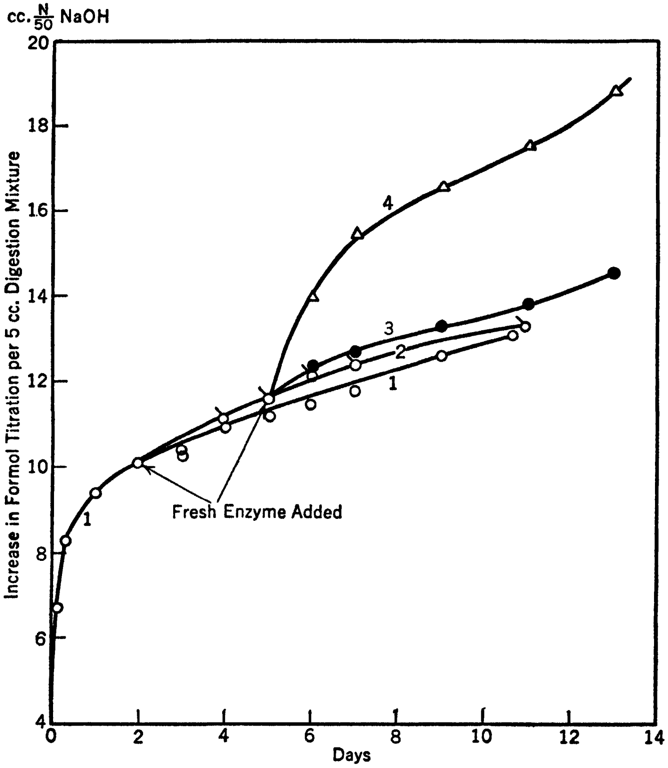


FIG. 17.—Digestion of casein by trypsin followed by chymotrypsin

100 cc. $\begin{cases}\text{5 per cent casein } p\text{H 7.6 } (M/10 \text{ phosphate}) \\ \text{0.08 mg. trypsin nitrogen/cc} \end{cases}$ .... = No. 1

After 2 days 0.08 mg. crystalline trypsin nitrogen/cc. added to 75 cc. No. 1 (Total trypsin concentration 0.16 mg. nitrogen/cc.)...... = No. 2

After 5 days 0.08 mg. crystalline trypsin nitrogen/cc. added to 25 cc. No. 2 (Total trypsin concentration 0.24 mg. nitrogen/cc.)....... = No. 3

After 5 days 0.08 mg. chymotrypsin nitrogen/cc. added to 25 cc. No. 2 (Total enzyme concentration 0.16 mg. trypsin nitrogen/cc. plus 0.08 mg. chymotrypsin nitrogen/cc.) .... = No. 4

Digestion determined by formol titration.

ously treated with chymotrypsin (Fig. 16), or of chymotrypsin to casein previously treated with trypsin, results in a marked increase in hydrolysis (Fig. 17) (99).

For a summary of the properties of chymotrypsinogen, chymotrypsin, and crystalline trypsin, see Table XI.

Crystalline Trypsin Inhibitor and Crystalline Inhibitor-Trypsin Compound

The activity of trypsin is influenced markedly by the presence of a substance in pancreatic extracts which inhibits trypsin digestion. This substance is present in the mother liquor from the trypsinogen crystallization. It has been isolated in a pure and crystalline state, and as a compound with trypsin by Northrop and Kunitz (100*a*, 100*b*). The inhibitor trypsin compound has also been crystallized. A scheme for the isolation of the two compounds is given in Table XII. The trypsin inhibitor compound contains approximately 80 per cent trypsin and 20 per cent inhibitor. In acid solution the trypsin is liberated and if added to protein solutions at *p*H 8.0 the protein is digested. No digestion of the protein will take place if the compound is neutralized before it is added to the protein solution. The inhibitor has a molecular weight of about 5,000 and appears to be a polypeptide.

In the case of rennin and pepsin, however, the present author (44) has shown that powerful inhibitors of these enzymes may be prepared by simply adding an equal volume of ethyl alcohol to the neutral solutions of their precursors.

Autolytic Trypsin

Kleiner and Tauber (101) autolyzed fresh ground pancreatic tissue in 30 per cent ethyl alcohol for eighteen months. After dialysis and acetone precipitation, the water-soluble fraction was dried in vacuum. A solution of this dry preparation gave a slightly positive xanthoproteic test, a

TABLE XI

Summary of the Properties of Chymotrypsinogen, Chymotrypsin, and Crystalline Trypsin (99)

		Chymotrypsinogen	Chymotrypsin	Trypsin
Crystalline form		Long, square prisms	Rhombohedrons	Short prisms
Elementary analysis per cent dry weight	Carbon	50.6	50.0	50.0
	Hydrogen	7.0	7.06	7.1
	Nitrogen	15.8	15.5	15.0
	Chlorine	0.17	0.16	2.85
	Sulfur	1.9	1.85	1.1
	Phosphorus	0	0	0
	Ash	0.1	0.12	1.0
Amino nitrogen as per cent total nitrogen	By formol	4.7	6.0	9.3
	By Van Slyke	4.75	6.0	
Tyrosine + tryptophane equivalent milliequivalents/mg. total nitrogen		2.5×10^{-3}	2.7×10^{-3}	3×10^{-3}
Optical activity, 25°		In 0.1 *M* acetic acid		*p*H 4.0 in 0.25 sat. ammonium sulfate
$[\alpha]_D$ line, per mg. nitrogen		−0.48	−0.40	−0.27
Solubility in distilled water		Slight	Very soluble	Very soluble
		In 0.5 *M* K_2SO_4, 0.1 *M* acetate *p*H 4.0		In 0.5 sat. $MgSO_4$
Diffusion coefficient, 6° cm.²/day	By nitrogen	0.039	0.037	0.023
	By hemoglobin	....	0.039	
	By rennet	....	0.037	
Molecular volume from diffusion coefficient, cm.³/mol.		52,000	52,000	65,000
Molecular weight from osmotic pressure		36,000 (32,000)	41,000	36,500
Hydration, grams water/grams protein, from osmotic pressure and diffusion coefficient		0.7	0.5	0.8
By viscosity		0	0.1	0.6
Isoelectric point from cataphoresis of collodion particles		5.0	5.4	7–8
	Substrate			
Specific activity [T. U.] per mg. protein nitrogen	Hemoglobin	$<1 \times 10^{-5}$	0.04	0.17
	Casein, sol.	$<1 \times 10^{-5}$	1.0	2.4
	Casein, F	<0.01	0.08	0.18
	Gelatin V	<0.1	12.0	100
	Rennet	<0.01	8.5	<0.1
	Clot blood	<2.0	<2.0	1500
	Sturin F	$<2 \times 10^{-4}$	0.018	0.63
*p*H optimum for digestion casein		....	8–9	8–9
Total digestion casein, cc. *M* 50 sodium hydroxide/5 cc. 5 per cent casein		....	17	9–11

TABLE XII

SCHEME FOR THE ISOLATION OF TRYPSIN INHIBITOR AND INHIBITOR-TRYPSIN COMPOUND

Filtrate from trypsinogen crystals
1 liter

Precipitate with magnesium sulfate at *p*H 3.0 and then with hot trichloroacetic acid.

Filter

Filtrate = crude inhibitor solution

Precipitate with magnesium sulfate at *p*H 3.0. Dissolve precipitate at *p*H 8.0 and add 1 gram crystalline trypsin. Adjust to *p*H 5.5, saturate with magnesium sulfate, 2 days at 20° C.

Filter

Precipitate = crystals inhibitor-trypsin compound
and amorphous material, 5 grams

Wash with 0.5 saturated magnesium sulfate; precipitate = inhibitor-trypsin compound. Recrystallize at *p*H 5.5 from saturated magnesium sulfate.

Crystalline inhibitor-trypsin compound
1 gram

Dissolve in water and precipitate with cold trichloroacetic acid.

Filter

Precipitate denatured trypsin

Dissolve in 0.02 *N* hydrochloric acid, fractionate with ammonium sulfate.

Filter

Precipitate amorphous trypsin

Crystallize from magnesium sulfate at *p*H 8.0 and 5° C.

Trypsin crystals, 0.25 gram

Filtrate solution of inhibitor

Heat to 80° C., filter. Saturate with magnesium sulfate at *p*H 3.0, filter. Dissolve precipitate at *p*H 5.5 and saturate with magnesium sulfate.

Inhibitor—crystals
0.15 gram

Inhibitor-trypsin compound

positive Folin-Denis test, and a positive ninhydrin test. The biuret test, the Millon test, and the Hopkins-Cole test were negative. The heat-coagulation test on prolonged boiling was slightly positive. Saturated solutions of neutral salts gave a precipitate. The isoelectric point of this trypsin was 6.2. It dialyzes readily through cellophane.

These properties are those of a polypeptide. The proteolytic activity of this trypsin is about that of crystalline trypsin. Its rennet activity was 1 : 420 as compared to 1 : 4,550,000, the potency of the highly active rennin of Tauber and Kleiner. It has been pointed out that proteolytic activity of trypsin must be depressed when the rennet activity is determined; otherwise, casein is digested without calcium paracaseinate formation. This preparation showed no amylolytic or lipolytic activity (102). A trypsin of similar nature has been obtained by Willstätter and Rohdewald (see above).

Papainases (Kathepsin and Papain)

Papainases are plant proteinases and are present in the cells of many plants. Papain is typical of this group. It is found in large amounts in the fruit and milky juice of the melon tree (*Carica papaya*). Kathepsin is the corresponding proteinase of animal cells. They have an optimum *p*H from 4 to 7. These two groups of proteases are very similar to each other. Considering the existing evidence, however, it is not difficult to draw a definite line between them. A labile SH group has been definitely established in the papain molecule. For urease the same is true.

The protein-liquefying property of papain has been known for many years. Vines, without knowing the reason for it (103), found that HCN, if added to papain, increases its activity. Mendel and Blood found that the HCN plays the rôle of an activator and that H_2S may be used instead of HCN. The early experiments of Mendel and Blood definitely showed that the HCN does not simply remove heavy metals. It is a specific activator. This was later verified by Willstätter and Grassmann (104).

If the HCN is removed by vacuum distillation or aeration, the initial slight activity is retained, and with the addition of more HCN, full activity may be obtained again.

An attempt at concentration of the enzyme by the adsorption procedure resulted only in a potency increase five times that of the original (Willstätter and Grassmann).

It has been shown that glutathione activates papain (105); likewise other SH compounds (106). According to Bersin and Logemann (107), the activation of papain by glutathione is due to the reduction of the oxidized S–S form, since active papain treated with hydrogen peroxide and subsequent reduction with H_2S became active again. Sodium sulfite and reduced glutathione may be used instead of H_2S for the reduction after hydrogen peroxide oxidation. These experiments were extended by Bersin (108), who has shown that ultra-violet irradiation may also produce activation. This is explained by the fact that cystine, treated similarly, produces cysteine. Papain may be activated also by organic and inorganic arsenic compounds which have the ability to reduce the S–S groups to the SH. Ascorbic acid does not activate papain, since it does not form thiol derivatives from disulfides.

Kathepsin, however, is activated by ascorbic acid (109). Karrer and Zehender (110) freed liver kathepsin from its natural activators and were able to reactivate it with ascorbic acid (see also Mashmann and Helmert, 111, 112).

The property of papain and kathepsin of being activated by sulfhydryl compounds has been made a basis by Purr and Russel (113) for the determination of such derivatives in blood cells. This work will be discussed below.

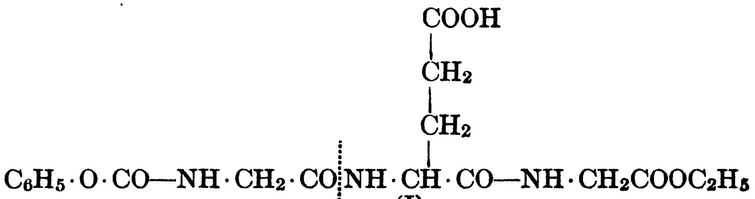
Grassmann (114) reported on the isolation of a natural activator (phytokinase) from papain preparations, which is not identical with glutathione. The activator is a peptide containing mostly cystine and glutamic acid.

Kathepsin is present in leucocytes, together with some tryptase (115–117). The cerebrospinal fluid of paralytic patients contains also kathepsin but no tryptase (118). With the proper technic, kathepsin may be detected by the presence of tryptase in blood serum also (119).

Krebs (120) tabulated the proteolytic activity of various organs at a slightly acid *p*H in the presence of cysteine, as follows: kidney, spleen, liver, lungs, testicles, heart muscle, and skeletal muscle.

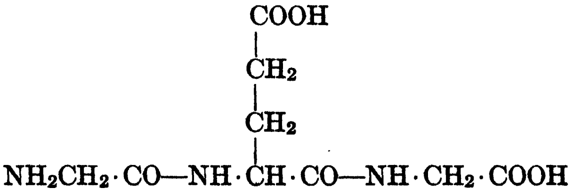
The Specificity of Papain

Bergmann, Zervas, and Fruton (121) recently made some remarkable observations concerning the specificity of papain. This proteinase requires two acid amide linkages. One is split, whereas the other appears to be necessary as a point of attachment for the enzyme. Unlike the peptidases (dipeptidase, aminopeptidase, and carboxypeptidase), papain does not require a free α-carboxyl or free amino group near the peptide linkage in the polypeptide molecule. Moreover, a neighboring free amino group inhibits papain activity. Carbobenzoxyglycylglutamylglycine ethyl ester (I) and carbobenzoxyglycylglutamylglycine are hydrolyzed to about 50 per cent in six hours; glycylglutamylglycine (II) is not attacked within this time.



$$C_6H_5\cdot O\cdot CO\text{—}NH\cdot CH_2\cdot CO\,\vdots\,NH\cdot \overset{\begin{array}{c}COOH\\ |\\ CH_2\\ |\\ CH_2\\ |\end{array}}{CH}\cdot CO\text{—}NH\cdot CH_2COOC_2H_5$$

(I)



$$NH_2CH_2\cdot CO\text{—}NH\cdot \overset{\begin{array}{c}COOH\\ |\\ CH_2\\ |\\ CH_2\\ |\end{array}}{CH}\cdot CO\text{—}NH\cdot CH_2\cdot COOH$$

(II)

A large number of acylated peptides have been found to be digestible by papain, whereas proteinases in general do not split peptides. The findings of Bergmann and associates (121), that papain-HCN at *p*H 5 does not hydrolyze the known substrates of peptidases, are contrary to those of Willstätter and Grassmann (104), who showed that tripeptides are hydrolyzed by papain-HCN. At the present no conclusions can be drawn concerning the hydrolysis of the above polypeptides by papain and the relation of these findings to the structure of the protein molecule. It should also be noted that it is not known whether papain is a single enzyme or a mixture of two or more enzymes.

Methods for the Estimation of Papain and Kathepsin

The activity of papain and other plant proteases may be estimated by methods used for the determination of other proteases (see above). Gelatin is a good substrate, and the optimum *p*H with it is 5.0 at 40°. The velocity of reaction decreases with the time. The activation of papain with HCN or H_2S is rapid, being completed in thirty to sixty minutes at 37°.

THE PAPAIN-ACTIVATING POWER OF BLOOD IN CANCER

The fact that papain and kathepsin can be activated by sulfhydryl compounds has been employed by A. Purr and M. Russel (113) for the determination of such compounds in blood cells, thus enabling them to study the intracellular metabolism in health and disease. First, they estimated papain activation by small amounts of glutathione and cysteine HCl. For example, 0.75 mg. glutathione in 10 cc. added to 2 cc. of an aqueous extract (1 : 50) of papain at *p*H 7.0 and 37° C. resulted in an increase in KOH (0.05 *N*) titration from 1 cc. to 1.5 cc. and from 1 cc. to 1.6 cc. in the case of cysteine using 8 per cent gelatin as substrate at *p*H 5.0. A standard curve has been worked out from which the degree of activation of the unknown can be read.

Purr and Russel found that the papain-activating power of the blood of patients suffering from cancer was markedly decreased when compared with normal blood. Sarcomatous rat's blood showed similar changes. The activating substances are present only in the formed elements of the blood (see Table XIII). In humans, the activating power falls below 50 per cent in all cases studied and is more marked than in rats. Since the amount of glutathione in blood is very small, another activating system must be present.

Papainase activation by blood deserves further consideration. The papain-activating power of sera in other diseases than cancer may be of diagnostic value.

PEPTIDASES

According to the early literature (122–124), "intestinal erepsin" can hydrolyze polypeptides, peptones of peptic and tryptic digestion, protamins, histones, and casein. Waldschmidt-Leitz and Schäffner (125), however, have shown that these statements were incorrect, since only peptides are attacked by the "intestinal erepsin." As has been stated above, in 1925 Waldschmidt-Leitz and associates separated "erepsin," the peptide-hydrolyzing fraction, from "pancreatic trypsin." In 1926, Willstätter and Grassmann (126) separated the proteolytic enzymes of yeast from the peptidase fraction, using an adsorption method similar to the one used by Waldschmidt-Leitz and Harteneck for the fractionation of pancreatic proteases (proteinase + carboxypolypeptidase). Now a number of specific polypeptidases and dipeptidases are known.

Polypeptidases

1. Aminopolypeptidases. The aminopolypeptidases attack the amino acid which is on the amino end of the polypeptide chain (see *A* in scheme below); i.e., the amino acid leucine is liberated. The amino group must be free

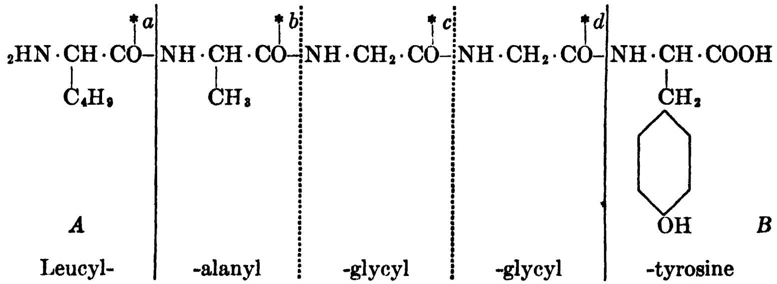
TABLE XIII

Papain Activation as a Measure of Physiologically Active Substances in Blood

Origin of blood	Activation effect in cc. 0.05 *N* KOH			Activation effect in milligrams active SH		Activation effect in mg. % active SH		Blood cells	
	Blood	Blood cells	Plasma	Blood	Blood cells	Blood	Blood cells	R.B.C. in million	W.B.C. in thousd.
Albino rats not inoculated	0.80	0.80	0.05	0.11	0.11	550	550	5.18	
	0.90	....	....	0.13	....	650	...	6.09	
	1.00	1.00	0.00	0.14	0.14	700	700	7.17	
	0.80	0.80	0.00	0.11	0.11	550	550	7.30	5.70
	0.75	....	....	0.09	....	450	...	6.55	6.40
	0.80	....	....	0.11	....	550	...	6.25	5.80
	1.25	....	....	0.18	....	900	...	9.06	8.09
	0.95	1.00	0.05	0.14	0.14	700	700	9.20	8.40
	1.25	....	....	0.18	....	900	...	8.80	5.50
	0.85	....	....	0.12	....	600			
	0.90	....	....	0.13	....	650			
Albino rats inoculated with sarcoma	0.80	....	....	0.11	....	550	...	5.96	
	0.50	0.50	0.00	0.07	0.07	350	350	6.29	
	0.50	0.60	0.05	0.07	0.08	350	400	10.78	
	0.60	....	....	0.08	....	400			
	0.50	....	....	0.07	....	350	...	7.47	13.40
	0.45	....	....	0.06	....	300	...	5.78	4.20
	0.45	....	....	0.06	....	300	...	7.73	28.40
	0.65	0.70	0.00	0.09	0.09	450	450	9.09	21.70
	0.80	0.60	0.05	0.11	0.08	550	400	6.11	9.11
	0.70	....	....	0.10	....	500	...	5.62	23.60
Healthy Man	0.90	....	....	0.13	....	325	...	5.14	7.70
	1.00	1.00	0.05	0.14	0.14	350	350	4.55	8.10
	1.00	1.00	0.00	0.14	0.14	350	350	4.79	3.70
	0.90	....	....	0.13	....	325			
	0.95	....	....	0.14	....	350			
Cancerous man	0.45	0.50	0.00	0.06	0.07	150	175	4.84	9.50
	0.40	....	....	0.06	....	150	...	5.24	7.50
	0.35	....	....	0.05	....	125	...	3.05	3.70
	0.50	0.45	0.00	0.07	0.06	175	150	3.93	10.40
	0.40	0.50	0.00	0.06	0.07	150	175	4.52	3.99
	0.45	....	....	0.06	....	150			

since peptide derivatives which have no free amino or carboxyl group are not hydrolyzed by peptidases. Balls and Köhler (127) described one instance.

In 1927, Grassmann and Dyckerhoff (128) reported on the fractionation of the ereptic mixture of yeast into a dipeptidase and polypeptidase component, and in 1930 Waldschmidt-Leitz and Balls (129) were able to separate the aminopolypeptidase of the intestine and other organs from the dipeptidase by adsorption of the dipeptidase on certain ferric hydroxides. A highly active preparation of protein nature and a considerable amount of organic P has been obtained by Balls and Köhler (130). Peptides with more than three amino acids and polypeptide esters are readily hydrolyzed by this enzyme.

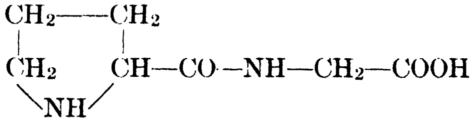


This scheme shows the various ways in which a polypeptide (leucylalanylglycylglycyltyrosine) may be hydrolyzed by peptidases. Thus leucine **a* or tyrosine **d* may be split off first, or a hydrolysis into di- and tripeptide may take place (**b* and **c*).

2. Pancreatic Polypeptidase. Pancreatic polypeptidase is probably a mixture of several enzymes. All of them, however, attack the amino acid which is on the carboxyl end of the polypeptide chain (see *B* in scheme). Abderhalden and Schwab (131) differentiate between a *leucinpeptidase* and a *tyrosinpeptidase,* which are supposed to be specific for splitting of leucin or tyrosin from polypeptides. In addition, the latter enzymes of the pancreas contain also an admixture of several acylimino acids and acylpeptides splitting acylases (132).

3. Prolinase or Prolylpeptidase. According to Grassmann, Dyckerhoff, and Schoenebeck (133), if the amino group of the α-amino acid of the peptide chain is replaced by the amino group of *prolin,* a specific polypeptidase, a so-called prolinase, acts upon it. Abderhalden and Zumstein (134, 135) found that not all prolylpeptides can be hydrolyzed by this intestinal enzyme. Recently, Grassmann and coworkers (136, 137) have taken up the study of this peptidase again and found that any prolylpeptide is split by the prolinase. They state that the polypeptides of Abderhalden and Zumstein were not free of protein, were not pure crystalline compounds, and were probably contaminated with silver salts, which were used for the preparation of the peptides, which had powerful inhibitory properties.

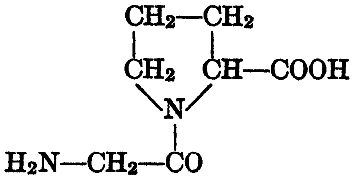
An example is



Prolylglycin

Prolinase may be obtained free from aminopolypeptidase but not from dipeptidase. The optimum *p*H is 7.8 when prolylglycin is used as a substrate.

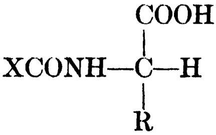
Recently, Bergmann and associates (138–140) have shown that in compounds in which the cyclic nitrogen of the prolin combines with the carboxyl group of a peptide, as, for example, in glycylprolin, enzymic hydrolysis may take place. This is in support



of the theory that proteins may be built of cyclic compounds instead of peptides. Contrary to Grassmann, Bergmann believes that this reaction is due to the aminopolypeptidase and not a special enzyme. In this glycylproline hydrolysis the imino group of proline is set free. This group cannot be estimated by the Van Slyke method. Here the law of equivalence is invalid.

Carboxypolypeptidase

According to Waldschmidt-Leitz and Purr (141), pancreatic carboxypolypeptidase hydrolyzes only a few dipeptides. It readily splits polypeptides and peptic digests in general. Bergmann, Zervas, and Schleich (142) showed that a substrate for carboxypolypeptidase must have the following configuration and groupings:



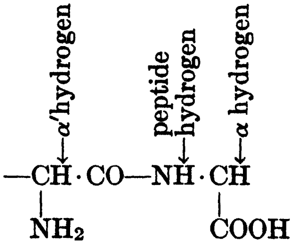
$$\begin{array}{rcl} & \mathrm{COOH} & \\ & | & \\ \mathrm{XCONH}— & \mathrm{C} & —\mathrm{H} \\ & | & \\ & \mathrm{R} & \end{array}$$

Crystalline Carboxypolypeptidase. Carboxypolypeptidase has been recently isolated from bovine pancreas by Anson (143) in crystalline form. It is a water-insoluble protein. It hydrolyzes acetyltyrosin and peptic digests.

Method of Crystallization. To the spontaneously activated fluid which exudes from frozen pancreas at 5°, 5 *N* acetic acid is added until brown cresol green turns green. The acid fluid is kept at 37° for two hours and filtered. The filtrate is diluted with ten times its volume of water. The precipitate which settles is filtered and suspended in water so as to give an activity twice the original fluid. Now $Ba(OH)_2$ is added, until the suspension is pink to phenolphthalein. The $Ba(OH)_2$ dissolves only a part of the protein but all the carboxypolypeptidase. NaOH dissolves all protein matter. The undissolved protein has been removed by centrifuging, and to the supernatant fluid 1 *N* acetic acid is added, until orange to phenol red. Globulin crystals appear on short standing. The protein enzyme can be dissolved in NaOH and recrystallized by neutralization. Recrystallization does not yield a less active enzyme, and destruction due to coagulation by heating is proportional to the protein coagulated. This enzyme digests peptic digest even in the presence of formaldehyde, proving that the presence of the free amino groups of neither enzyme nor substrate is essential for carboxypolypeptidase activity. Other pancreatic enzymes do not act in the presence of formaldehyde.

Dipeptidases

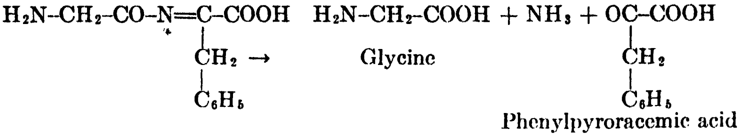
1. Dipeptidase. The dipeptidase of "erepsin" has been well studied. It is found in the erepsin fraction of pancreatic tissue and other tissues associated with aminopolypeptidase. According to Bergmann and Zervas (144, 145) a dipeptid to be hydrolyzed by a peptidase must contain natural α-amino acids, a normal peptide linkage, a free carboxyl group bound to an adjacent carbon atom, and an amino group similarly attached (see Scheme):



$$—\underset{\mathrm{NH_2}}{\overset{\alpha'\text{ hydrogen}\downarrow}{\mathrm{CH}}}\cdot\mathrm{CO}—\mathrm{N}\overset{\text{peptide hydrogen}\downarrow}{\mathrm{H}}\cdot\underset{\mathrm{COOH}}{\overset{\alpha\text{ hydrogen}\downarrow}{\mathrm{CH}}}$$

There must be at least one free hydrogen atom on the peptide linkage nitrogen, and at least one hydrogen on the α carbon atom and one on the α' carbon atom. The α and α' carbons must have the proper configuration. Opinions differ, however, concerning the absolute specificity of dipeptidases (146–150).

2. Dehydropeptidase. Dehydropeptidase splits substances like glycyldehydrophenylalanin as follows:



$$\underset{\substack{| \\ CH_2 \\ | \\ C_6H_5}}{H_2N\text{-}CH_2\text{-}CO\text{-}N{=}C\text{-}COOH} \rightarrow \underset{\text{Glycine}}{H_2N\text{-}CH_2\text{-}COOH} + NH_3 + \underset{\substack{| \\ CH_2 \\ | \\ C_6H_5 \\ \text{Phenylpyroracemic acid}}}{OC\text{-}COOH}$$

It is present in large quantities in autolytic kidney and pancreas tissues. It is not present, however, in other organs. This enzyme is a *dipeptidase.* It is very sensitive to HCN. Its optimum *p*H is 7.5 (151, 152).

Bromelin, Keratinase, and Other Proteolytic Enzymes

Bromelin is the proteinase of the pineapple. It is very similar to papain and is also activated by HCN (153, 154). Squash too contains a *papain-like proteinase* but it is inhibited by HCN and H_2S (132). Peptidases accompany both proteolytic enzymes. *A proteinase with an alkaline optimum* *p*H has been obtained by Tauber and Kleiner (155) from the fruits of *Solanum indicum.* Interesting is the reported isolation of a pepsin-like enzyme from cucumber by Chopra and Roy (156). This proteinase is activated by HCl and citric acid. Such enzymes are not found usually in the plant kingdom. It digests peptone and casein, liquefies gelatin, clots milk, but does not digest fibrin. Its optimum *p*H is between 5 and 6.

Maschmann (157) has recently published an extensive study concerning plant proteases such as papain, bromelin, and the yeast proteinase.

Keratinase, a specific keratin-hydrolyzing enzyme, may be obtained, according to Schultz (158), from the clothes moth. Other proteolytic enzymes do not digest keratin. The enzyme has been reported to be present in the digestive tract of birds of prey (159). Mangold and Dubiski (160) believe, however, that there is not enough evidence for the existence of a specific keratinase.

Classification and Nomenclature of Proteolytic Enzymes and Peptidases

The early division of all proteolytic enzymes into pepsins, trypsins, and erepsins has been found to be inadequate. Proteolytic enzymes may be divided into two main classes: proteinases and peptidases. The proteinases hydrolyze proteins, including conjugated proteins, to proteoses, peptones, and polypeptides respectively, whereas the peptidases split peptides.

I. *Proteinases*

1. Pepsinases (gastric pepsins).
2. Tryptases (various pancreatic trypsins, and plant trypsins).
3. Papainases (papains of plant tissues and kathepsins of animal tissues).
4. Protaminases (hydrolyze protamines, peptone-like substances).
5. Other proteolytic enzymes (not belonging to any of the above groups).

II. *Polypeptidases*

1. Aminopolypeptidases (of small intestine, pancreas, spleen, and other organs, as well as blood serum and blood cells).
2. Pancreatic carboxypolypeptidase.
3. Prolynase.

III. *Dipeptidases*

1. Dipeptidases (possibly associated with some polypeptidases).
2. Dehydropeptidase.

REFERENCES

1. Waldschmidt-Leitz, E., Schäffner, A., and Grassmann, W.: Über die Struktur des Clupeins (Erste Mitteilung über enzymatische Proteolyse). *Z. physiol. Chem.*, **156**, 68 (1926).

2. Waldschmidt-Leitz, E., and Simons, E.: Über die enzymatische Hydrolyse des Caseins (Zweite Mitteilung über enzymatische Proteolyse). *Ibid.*, **156**, 99 (1926).
3. Waldschmidt-Leitz, E., and Simons, E.: Über die Wirkungsweise des Pepsins. (Sechste Mitteilung zur Spezifität tierischer Proteasen). *Ibid.*, **156**, 114 (1926).
4. Waldschmidt-Leitz, E., and Künstner, G.: Zur Kenntnis der Pepsinwirkung (Elfte Mitteilung zur Spezifität tierischer Proteasen). *Ibid.*, **171**, 70 (1927).
5. Waldschmidt-Leitz, E., and Künstner, G.: Fraktionierte enzymatische Hydrolyse des Histons (Vierte Mitteilung über enzymatische Proteolyse). *Ibid.*, **171**, 290 (1927).
6. Grassmann, W., and Dyckerhoff, H.: Über die Proteinase und die Polypeptidase der Hefe. 13 Abhandlung über Pflanzenproteasen in der von R. Willstätter und Mitarbeitern begonnenen Untersuchungsreihe. *Z. physiol. Chem.*, **179**, 41 (1928).
7. Weber, H. H., and Gesenius, H.: Proteasen und proteolytische Hemmungskörper. *Biochem. Z.*, **187**, 410 (1927).
8. Felix, K., and Harteneck, A.: Über den Aufbau des Histons der Thymusdrüse. Dritte Mitteilung. Das Säuren- und Basenbindungsvermögen nach Pepsinverdauung. *Z. physiol. Chem.*, **165**, 103 (1927).
9. Sörensen, S. P. L., and Katschioni-Walther, L.: Über die Pepsinspaltung. Mit einer Notiz über die Indicatorfrage von Linderstromlang, K. *Z. physiol. Chem.*, **174**, 251 (1928).
10. Abderhalden, E., and Kröner, W.: Vergleichende Studien über den Abbau von Casein, Serumglobulin und Serumalbumin durch verdünntes Alkali, verdünnte Säure, Pepsinsalzsäure und Pankreasfermente. *Fermentforschung*, **10**, 12 (1928–29).
11. Vickery, H. B., and Osborne, T. B. A review of hypotheses of the structure of proteins. *Physiol. Rev.*, **8**, 393 (1928).
12. Richardson, G. M.: The principle of formaldehyde, alcohol, and acetone titrations. With a discussion of the proof and implication of the Zwitterion conception. *Proc. Roy. Soc. London, B*, **115**, 121 (1934).
13. *Idem:* Critique on the biological estimation of amino nitrogen. *Ibid.*, *B*, **115**, 142 (1934).
14. Abderhalden, E.: Das Eiweiss als eine Zusammenfassung Assoziierter, Anhydride enthaltender Elementarkomplexe. *Naturwissenschaften*, **12**, 716 (1924).
15. Abderhalden, E.: Über die Struktur der Proteine, *Z. physiol. Chem.*, **128**, 119 (1923).
16. Abderhalden, E., and Schwab, E.: Über die Anhydridstruktur des Seidenfibroins. *Z. physiol. Chem.*, **139**, 169 (1924).

17. Abderhalden, E., and Komm, E.: Über die Anhydridstruktur der Proteine. *Ibid.*, **139**, 181 (1924).

18. Abderhalden, E., and Schwab, E.: Studien über das Verhalten von Polypeptiden und von Kombinationen von 2.5-Dioxopiperazinen mit Aminosäuren, an deren Aufbau Serin beteiligt ist, gegenüber Fermenten und ferner in Lösungen verschiedener Wasserstoffionenkonzentration. *Z. physiol. Chem.*, **171**, 78 (1927).

19. Bergmann, M.: Über neurere Proteinchemie. *Naturwissenschaften*, **12**, 1155 (1924).

20. *Idem:* Über den hochmolekularen Zustand der Proteine und die Synthese proteinähnlicher Piperazin-Abkömmlinge. *Ibid.*, **13**, 1045 (1925).

21. Abderhalden, E., and Schwab, E.: Über Verbindungen vom Typus: Aminosäure (2-5-Dioxopiperazin) und ihre Verhalten gegenüber Säure Alkali und Fermenten. *Z. physiol. Chem.*, **212**, 61 (1932).

22. Matsui, J.: Konstitution der Polypeptide und proteolytische Fermenten. *J. Biochem.*, **17**, 163 (1933).

23. Matsui, J.: Konstitution der Polypeptide und proteolytische Fermenten. *J. Biochem.*, **17**, 253 (1933).

24. Ishiyama, T.: Über fermentative Aufschliessung des Diketopiperazinrings. *J. Biochem.*, **17**, 285 (1933).

25. Shibata, K.: Über die künstlichen Substrate für Proteinasen und über die Proteinstruktur. *Acta Phytochim.*, **8**, 173 (1934).

26. Tazawa, Y.: Über die Synthese des *d*-Argininanhydrids und des *d*-Lysinanhydrids und über ihre Ringspaltung durch Pepsin. *Acta Phytochim.*, **8**, 331 (1935).

27. Greenstein, J. P.: Studies on multivalent amino acids and peptides. *J. Biol. Chem.*, **112**, 517 (1936).

28. Svedberg, Th., and Nichols, J. B.: The molecular weight of egg albumin. I. In electrolyte-free condition. *J. Am. Chem. Soc.*, **48**, 3081 (1926).

29. Svedberg, Th., and Sjögren, B.: The molecular weights of serum albumin and of serum globulin. *Ibid.*, **50**, 3318 (1928).

30. Svedberg, Th., and Heyroth, F. F.: The molecular weight of the hemocyanin of *Limulus polyphemus*. *Ibid.*, **51**, 539 (1929).

31. *Idem:* The influence of the hydrogen-ion activity upon stability of the hemocyanin of *Helix pomatia*. *Ibid.*, **51**, 550 (1929).

32. Svedberg, Th., and Stamm, A. J.: The molecular weight of edestin. *J. Am. Chem. Soc.*, **51**, 2170 (1929).

33. Svedberg, Th.: Über die Bestimmung von Molekulargewichten durch Zentrifugierung. *Z. physiol. Chem.*, **121**, 65 (1926).

34. Svedberg, Th.: Mass and size of protein molecules. *Nature*, **123**, 871 (1929).

35. Goddard, D. R., and Michaelis, L.: A study on keratin. *J. Biol. Chem.*, **106**, 605 (1934).
36. Schwann, T.: Über das Wesen des Verdauungsprozesses. *Müller's Archiv*, **90** (1836).
37. Northrop, J. H.: The presence of a gelatin-liquefying enzyme in crude pepsin preparations. *J. Gen. Physiol.*, **15**, 29 (1931–32).
38. Holter, H.: Zur Kenntnis des Pepsins. Vorläufige Mitteilung. *Z. physiol. Chem.*, **196**, 1 (1931).
39. Willstätter, R., and Rohdewald, M.: Über Desmo-Pepsin und Desmo-Kathepsin (Erste Abhandlung. Zur Kenntnis zellgebundener Enzyme der Gewebe und Drüsen). *Z. physiol. Chem.*, **208**, 258 (1932).
40. Tauber, H., and Kleiner, I. S.: Studies on rennin. 1. The purification of rennin and its separation from pepsin. *J. Biol. Chem.*, **96**, 745 (1932).
41. Kleiner, I. S., and Tauber, H.: Further studies of zymogens of pepsin and rennin. *J. Biol. Chem.*, **106**, 501 (1934).
41*a*. Herriott, R. M., and Northrop, J. H.: Isolation of crystalline pepsinogen from swine gastric mucosae and its autocatalytic conversion into pepsin. *Science*, **83**, 469 (1936).
42. Northrop, J. H.: Crystalline pepsin. III. Preparation of active crystalline pepsin from inactive denatured pepsin. *J. Gen. Physiol.*, **14**, 713 (1930–31).
43. Northrop, J. H.: Crystalline pepsin. I. Isolation and tests of purity. *J. Gen. Physiol.*, **13**, 739 (1929–30).
44. Tauber, H.: Inhibitors of milk-curdling enzymes. *J. Biol. Chem.*, **107**, 161 (1934).
45. Holter, H., and Andersen, B.: Vergleich der Pepsin- und Labaktivität verschiedener Magensekrete. *Biochem. Z.*, **269**, 285 (1934).
46. Hammarsten, O.: Vergleichende Untersuchungen über Pepsin- und Chymosin-wirkung bei Hund und Kalb. *Z. physiol. Chem.*, **68**, 119 (1910).
47. Northrop, J. H.: Crystalline pepsin. V. Isolation of crystalline pepsin from bovine gastric juice. *J. Gen. Physiol.*, **16**, 615 (1932–33).
48. Hedin, S. G.: Über das Labzymogen des Kalbsmagens. *Z. physiol. Chem.*, **72**, 187 (1911).
49. Linderstroem-Lang, K.: Über die Einheitlichkeit des Kaseins. Vorläufige Mitteilung. *Z. physiol. Chem.*, **176**, 76 (1928).
50. Linderstroem-Lang, K.: Studies on casein. III. On the fractionation of casein. *Compt. rend. trav. lab. Carlsberg*, **17**, No. 9 (1928).
51. Tauber, H., and Kleiner, I. S.: Studies on trypsin. II. The effect of trypsin on casein. *J. Biol. Chem.*, **104**, 271 (1934).

52. Clifford, W. M.: The effect of halogen salts on the clotting of milk by trypsin. *Biochem. J.*, **29**, 1059 (1935).

53. Northrop, J. H.: Crystalline pepsin. I. Isolation and tests of purity. *J. Gen. Physiol.*, **13**, 739 (1929–1930).

54. Pekelharing, C. A.: Mitteilungen über Pepsin. *Z. physiol. Chem.*, **358** (1902).

55. Herriott, R. M., and Northrop, J. H.: Crystalline acetyl derivatives of pepsin. *J. Gen. Physiol.*, **18**, 35 (1934).

56. Sörensen, S. P. L.: Enzymstudien (Zweite Mitteilung über die Messung und die Bedeutung der Wasserstoffionenkonzentration bei enzymatischen Prozessen). *Biochem. Z.*, **21**, 288 (1909).

57. Michaelis, L.: Die Wasserstoffionenkonzentration. Berlin. *Monogr. Physiol. Pflanzen*, **1** (1914).

58. Pekelharing, C. A., and Ringer, W. E.: Zur elektrischen Überführung des Pepsins. *Z. physiol. Chem.*, **75**, 282 (1911).

59. Euler, H. von. Fermentative Spaltung von Dipeptiden. *Z. physiol. Chem.*, **51**, 213 (1907).

60. Arrhenius, S.: Quantitative Laws in Biological Chemistry. 1915.

61. Northrop, J. H.: The mechanism of the influence of acids and alkalis on the digestion of proteins by pepsin or trypsin. *J. Gen. Physiol.*, **5**, 263 (1922).

62. Linderstroem-Lang, K.: Titrimetrische Bestimmung von Aminostickstoff. *Z. physiol. Chem.*, **173**, 32 (1928).

63. Holter, H.: Zur Kenntnis des Pepsins. *Z. physiol. Chem.*, **196**, 1 (1931).

64. Holter, H., Linderstroem-Lang, K., and Bronnicke Funder, J.: Über den peptischen Abbau des Caseins. *Z. physiol. Chem.*, **206**, 85 (1932).

65. Andersen, B.: The determination of pepsin and rennin activity in gastric juice. *Compt. rend. trav. lab. Carlsberg*, **19**, No. 19 (1933).

66. Willstätter, R., Waldschmidt-Leitz, E., Dunaiturria, S., and Künstner, G.: Zur Kenntnis des Trypsins. XV. Abhandlung über Pankreasenzyme. *Z. physiol. Chem.*, **161**, 191 (1926).

67. Van Slyke, D. D.: The manometric determination of urea in blood and urine by the hypobromite reaction. *J. Biol. Chem.*, **83**, 449 (1929).

68. Northrop, J. H.: Pepsin activity units and methods for determining peptic activity. *J. Gen. Physiol.*, **16**, 41 (1931–32).

69. Anson, M. L., and Mirsky, A. E., The estimation of pepsin with hemoglobin. *J. Gen. Physiol.*, **16**, 59 (1931–32).

70. Kühne, W.: Über die Verdauung der Eiweisstoffe durch den Pankreassaft. *Virchows Arch. path. Anat.*, **39**, 130 (1867).

71. Heidenhain, R.: Beiträge zur Kenntniss des Pankreas. *Arch. ges. Physiol.*, **10**, 557 (1875).

72. SCHEPOWALNIKOW, N. P.: Die Physiologie des Darmsaftes. *Jahresber. Fortschr. Tierchem.*, **29**, 378 (1900).

73. VERNON, H. M.: The conditions of conversion of pancreatic zymogens into enzymes. *J. Physiol.*, **27**, 269 (1901).

74. VERNON, H. M.: The auto-catalysis of trypsinogen. *J. Physiol.*, **47**, 325 (1913).

75. BAYLISS, W. M., and STARLING, E. H.: The proteolytic activities of the pancreatic juice. *J. Physiol.*, **30**, 61 (1904).

76. WALDSCHMIDT-LEITZ, E.: Über Enterokinase und die tryptische Wirkung der Pankreasdrüse (Fünfte Abhandlung über Pankreasenzyme) von Willstätter und Mitarbeitern. *Z. physiol. Chem.*, 132, 181 (1924).

77. VERNON, H. M.: Pancreatic zymogens and pro-zymogens. *J. Physiol.*, **28**, 448 (1902).

78. WILLSTÄTTER, R., and WALDSCHMIDT-LEITZ, E.: Über Pankreaslipase (Zweite Abhandlung über Pankreasenzyme). *Z. physiol. Chem.*, **125**, 132 (1923).

79. WILLSTÄTTER, R.: Problems and Methods in Enzyme Research. Published by Cornell University, Ithaca, N. Y., 1927.

80. WALDSCHMIDT-LEITZ, E., and HARTENECK, A.: Zur Kenntnis der spontanen Aktivierung des Trypsins (Vierte Mitteilung zur Spezifität tierischer Proteasen. *Z. physiol. Chem.*, **149**, 221 (1925).

81. WALDSCHMIDT-LEITZ, E., SCHÄFFNER, A., and GRASSMANN, W.: Über die Struktur des Clupeins (Erste Mitteilung über enzymatische Proteolyse). *Z. physiol. Chem.*, **156**, 68 (1926).

82. WALDSCHMIDT-LEITZ, E., and LINDERSTROEM-LANG, K.: Darstellung von enterokinasefreies Trypsin (Achte Mitteilung zur Spezifität tierischer Proteasen). *Z. physiol. Chem.*, **166**, 241 (1927).

83. ABDERHALDEN, E., and SCHWAB, E.: Weiterer Beitrag zum Problem der komplexen Natur des Trypsins. *Fermentforschung*, **12**, 559 (1930–31).

84. LINDERSTROEM-LANG, K.: Studies on proteolytic enzymes. IX. On the cleavage of leucyldecarboxyglycine by intestinal erepsin. *Compt. rend. trav. lab. Carlsberg*, **19**, No. 3 (1931).

85. WILLSTÄTTER, R., BAMANN, E., and ROHDEWALD, M.: Über das Trypsin der farblosen Blutkörperchen (Fünfte Abhandlung über Enzyme der Leukocyten). *Z. physiol. Chem.*, **188**, 107 (1930).

86. WILLSTÄTTER, R., and ROHDEWALD, M.: Über Desmo und Lyo-Trypsin der farblosen Blutkörperchen. *Z. physiol. Chem.*, **204**, 181 (1932).

87. WALDSCHMIDT-LEITZ, E., and AKABORI, S.: Über die enzymatischen Komponenten der Proteinase aus Pankreas. *Z. physiol. Chem.*, **228**, 224 (1934).

88. Waldschmidt-Leitz, E.: Zur Kenntnis der Enterokinase. *Z. physiol. Chem.*, **142**, 217 (1925).

89. Waldschmidt-Leitz, E., and Waldschmidt-Graser, J.: Über die enzymatischen Wirkungen von Pankreas- und Darmsecretion. *Z. physiol. Chem.*, **166**, 247 (1927).

90. Pace, J.: The formation of enterokinase from a precursor in the pancreas. A supplementary note. *Biochem. J.*, **26**, 1566 (1932).

91. Bates, R. W., and Koch, F. C.: Studies on the trypsinogen, enterokinase and trypsin system. Assay methods for trypsinogen and enterokinase. *J. Biol. Chem.*, **111**, 197 (1935).

92. Northrop, J. H.: Personal communication to the author.

93. Linderstroem-Lang, K., and Steenberg, E. M.: On the determination of trypsin and enterokinase. *Compt. rend. trav. lab. Carlsberg*, **17**, No. 16 (1929).

94. Bates, R. W.: A method for the determination of enterokinase. *Proc. Soc. Exptl. Biol. Med.*, **28**, 1055 (1931).

95. Willstätter, R., and Rohdewald, M.: Über pankreatisches Desmotrypsin (Zweite Abhandlung. Zur Kenntnis zellgebundener Enzyme der Gewebe und Drüsen). *Z. physiol. Chem.*, **218**, 77 (1933).

96. Northrop, J. H., and Kunitz, M.: Crystalline trypsin. *J. Gen. Physiol.*, **16**, 267 (1932).

97. Northrop, J. H., and Kunitz, M.: Crystalline trypsin. II. General properties. *J. Gen. Physiol.*, **16**, 295 (1932).

98. Pace, J.: The inactivation of trypsin by heat. *Biochem. J.*, **24**, 606 (1930).

99. Kunitz, M., and Northrop, J. H.: Crystalline chymo-trypsin and chymo-trypsinogen. I. Isolation, crystallization, and general properties of a new proteolytic enzyme and its precursor. *J. Gen. Physiol.*, **18**, 433 (1935).

100. Kunitz, M., and Northrop, J. H.: The isolation of crystalline trypsinogen and its conversion into crystalline trypsin. *Science*, **80**, 505 (1934).

100*a*. Kunitz, M., and Northrop, J. H.: Isolation from beef pancreas of crystalline trypsinogen, trypsin, a trypsin inhibitor, and an inhibitor-trypsin compound. *J. Gen. Physiol.*, **19**, 991 (1936).

100*b*. Northrop, J. H., and Kunitz, M.: Discussed by Northrop in: *The Harvey Lectures*, **30**, 229 (1934–35).

101. Kleiner, I. S., and Tauber, H.: Studies on trypsin. I. The chemical nature of trypsin. *J. Biol. Chem.*, **104**, 267 (1934).

102. The author's unpublished results.

103. Vines, S. H.: Annals of botany, proteolytic enzymes in plants. *Ann. Botany*, **17**, 237 (1903). The proteases of plants. I. *Ibid.*, **18**, 289 (1904). The proteases of plants. II. *Ibid.*, **19**, 149 (1905).

The proteases of plants. IV. *Ibid.*, **20**, 113 (1906). The proteases of plants. V. *Ibid.*, **22**, 103 (1908). The proteases of plants. VI. *Ibid.*, **23**, 1 (1909). The proteases of plants. VII. *Ibid.*, **24**, 213 (1910).

104. Willstätter, R., and Grassmann, W.: Über die Aktivierung des Papains durch Blausäure (Erste Abhandlung über pflanzliche Proteasen). *Z. physiol. Chem.*, **138**, 184 (1924).
105. Waldschmidt-Leitz, E., and Purr, A.: Über Zookinase. *Z. physiol. Chem.*, **198**, 260 (1931).
106. Waldschmidt-Leitz, E., Scharikova, A., and Schäffner, A.: Über den Einfluss von Sulfhydrylverbindungen auf enzymatische Prozesse. *Z. physiol. Chem.*, **214**, 75 (1933).
107. Bersin, Th., and Logemann, W.: Über den Einfluss von Oxydations und Reduktionsmitteln auf Papain. *Z. physiol. Chem.*, **220**, 209 (1933).
108. Bersin, Th.: Über die Einwirkung von Oxydations und Reduktionsmitteln auf Papain. II. Die Aktivitätsbeeinflussung durch Licht, Organoarsenverbindungen und Ascorbinsäure. *Z. physiol. Chem.*, **222**, 177 (1933).
109. Purr, A.: Influence of vitamin C on intracellular enzyme action. *Biochem. J.*, **27**, 1703 (1933).
110. Karrer, P., and Zehender, F.: Vitamin C (Ascorbinsäure) als Aktivator katheptischer Enzyme. I. *Helv. Chim. Acta*, **16**, 701 (1933).
111. Maschmann, E., and Helmert, E.: Über Hemmung des Kathepsins und Aktivierung des Papains durch Sulfhydrylcarbonsäuren. *Z. physiol. Chem.*, **222**, 207 (1933).
112. Maschmann, E., and Helmert, E.: Über die Aktivierung des "Papains" durch Vitamin C-Eisen und seine Hemmung durch Vitamin C (Ascorbinsäure). *Z. physiol. Chem.*, **223**, 127 (1934).
113. Purr, A., and Russel, M.: Die Aktivierungsfähigkeit des Papains, angewendet auf eine Bestimmungsmethode physiologisch aktiver Stoffe in Blut. *Z. physiol. Chem.*, **228**, 198 (1934).
114. Grassmann, W.: Zur Kenntnis des natürlichen Papain-Aktivators. *Biochem. Z.*, **279**, 137 (1935).
115. Husfeldt, E.: Proteolytische Enzyme in den Leukocyten des Menschen. *Z. physiol. Chem.*, **194**, 137 (1931).
116. Rona, P., and Kleinmann, H.: Untersuchungen über tierische Gewebsproteasen (Sechste Mitteilung). Über die Arten der in Lymphdrüsen vorhandenen Proteinasen. *Biochem. Z.*, **241**, 283 (1931).
117. Rona, P., and Kleinmann, H.: Untersuchungen über tierische Gewebsproteasen (Siebente Mitteilung). Qualitative und quantita-

tive Untersuchung des Kathepsins der Pferdelymphdrüsen. *Biochem. Z.*, **241**, 316 (1931).

118. Heyde, W.: Zur Kenntnis der Proteasen der Cerebrospinalflüssigkeit. *Z. ges. Neurol. Psych.*, **138**, 536 (1932).
119. Kleinmann, H., and Scharr, G.: Untersuchungen über tierische Gewebsproteasen (Zehnte Mitteilung). Über das Auftreten von Abwehrfermenten im Blutserum des Kaninchens. *Biochem. Z.*, **252**, 343 (1932).
120. Krebs, H. A.: Über die Proteolyse der Tumoren. *Biochem. Z.*, **238**, 174 (1931).
121. Bergmann, M., Zervas, L., and Fruton, J. S.: On proteolytic enzymes. VI. On the specificity of papain. *J. Biol. Chem.*, **111**, 225 (1935).
122. Cohnheim, O.: Die Verwandlung des Eiweiss durch die Darmwand. *Z. physiol. Chem.*, **33**, 451 (1901).
123. Kühne, W.: Über die Verdauung der Eiweisstoffe durch den Pankreassaft. *Virchow's Arch. path. Anat.*, **39**, 130 (1867).
124. Kühne, W.: Erfahrungen über Albumosen und Peptoine. I. *Z. Biol.*, **29**, 1 (1892); II. **29**, 308 (1892).
125. Waldschmidt-Leitz, E., and Schäffner, A.: Zur Kenntnis des Darmerepsins. *Z. physiol. Chem.*, **151**, 31 (1926).
126. Willstätter, R., and Grassmann, W.: Über die Proteasen der Hefe. *Z. physiol. Chem.*, **153**, 250 (1926).
127. Balls, A. K., and Köhler, F.: Über eine neue proteolytische Wirkung von Darmschleimhaut-Auszügen. *Ber.*, **64**, 383 (1931).
128. Grassmann, W., and Dyckerhoff, H.: Über die Spezifität der Hefe-Peptidasen. *Ber.*, **61**, 656 (1928).
129. Waldschmidt-Leitz, E., and Balls, A. K.: Zur Kenntnis der Amino-Polypeptidase aus Darm-Schleimhaut. *Ber.*, **63**, 1203 (1930).
130. Balls, A. K., and Köhler, F.: Über Aktivität und Phosphorgehalt von Amino-Polypeptidase. *Naturwissenschaften*, **19**, 737 (1931).
131. Abderhalden, E., and Schwab, E.: Weiterer Beiträge zur Frage der Einheitlichkeit des Trypsinkomplexes (Zweite Mitteilung. Versuche einer Abtrennung von Fermenten mit verschiedener Wirkung bei der Gewinnung von "Trypsin" aus Pankreaspulver). *Fermentforschung*, **10**, 478 (1929).
132. Abderhalden, E., and Ehrenwall, E.: Beiträge zur Frage der Einheitlichkeit des Erepsins. *Fermentforschung*, **12**, 223 (1930–31).
133. Grassmann, W., Dyckerhoff, H., and Schoenebeck, O. von: Über die enzymatische Spaltbarkeit der Prolin-Peptide. *Ber.*, **62**, 1307 (1929).
134. Abderhalden, E., and Zumstein, O.: Weiters Studien über das Verhalten von Prolin enthaltenden Polypeptiden gegenüber dem

Erepsin- und Trypsin-Kinase-Komplex. *Fermentforschung*, **12**, 341 (1930–31).

135. Abderhalden, E., and Zumstein, O.: Über das Verhalten von Prolin enthaltenden Polypeptiden gegenüber dem Erepsin- und dem Trypsin-Kinase-Komplex. *Fermentforschung*, **12**, 1 (1930–31).
136. Grassmann, W., Schoenebeck, O. von, and Auerbach, G.: Über die enzymatische Spaltbarkeit der Prolinpeptide. II. *Z. physiol. Chem.*, **210**, 1 (1932).
137. Grassmann, W., Schoenebeck, O. von, and Auerbach, G.: Berichtigung. *Z. physiol. Chem.*, **214**, 104 (1933).
138. Bergmann, M., Zervas, L., Schleich, H., and Leinert, F.: Über proteolytische Fermente, Verhalten von Prolinpeptiden. *Z. physiol. Chem.*, **212**, 72 (1932).
139. Bergmann, M.: Neue Synthesen und Enzymversuche im Eiweissgebiet. *Klin. Wochenschr.*, **11**, 1569 (1932).
140. Bergmann, M., Zervas, L., and Schleich, H.: Über proteolytische Enzyme (Zweite Mitteilung. Bindungsart des Prolins in der Gelatine). *Ber.*, **65**, 1747 (1932).
141. Waldschmidt-Leitz, E., and Purr, A.: Specifität tierischer Proteasen. XVII. Proteinase und Carboxypolypeptidase aus Pankreas. *Ber.*, **62**, 2217 (1929).
142. Bergmann, M., Zervas, L., Schleich, H.: Über proteolytische Enzyme. IV. Spezifität und Wirkungsweise der sogenannten Carboxypolypeptidase. *Z. physiol. Chem.*, **224**, 45 (1934).
143. Anson, M. L.: Crystalline carboxypolypeptidase. *Science*, **81**, 467 (1935).
144. Bergmann, M., and Zervas, L.: Über proteolytische Enzyme. *Z. physiol. Chem.*, **224**, 11 (1934).
145. Bergmann, M.: Synthesis and degradation of proteins in the laboratory and in metabolism. *Science*, **79**, 439 (1934).
146. Grassmann, W., and Dyckerhoff, H.: Über die Spezifität der Hefe-Peptidasen. *Ber.*, **61**, 656 (1928).
147. Balls, A. K., and Köhler, F.: Über den Mechanismus der enzymatischen Dipeptid-Spaltung. *Ber.*, **64**, 34 (1931).
148. Sato, M.: Studies of proteolytic enzymes. VII. On the peptidases. *Compt. rend. trav. lab. Carlsberg*, **19**, No. 1 (1931).
149. Bergmann, M., Schmitt, V., and Miekeley, A.: Über Peptide dehydrierter Aminosäuren, ihr Verhalten gegen pankreatische Fermente und ihre Verwendung zur Peptidsynthese. *Z. physiol. Chem.*, **187**, 264 (1930).
150. Bergmann, M., and Schleich, H.: Zur Spezifität der Peptidasen. *Naturwissenschaften*, **18**, 832 (1930).
151. Bergmann, M., and Schleich, H.: Über die enzymatische Spaltung

dehydrierter Peptide. Auffindung einer Dehydrodipeptidase. *Z. physiol. Chem.*, **205**, 65 (1932).

152. Bergmann, M., and Schleich, H.: Weiteres über Dehydro-dipeptidase. Über die enzymatische Angreifbarkeit von Verbindungen aus Brenztraubensäure und Aminosäure. *Z. physiol. Chem.*, **207**, 235 (1932).
153. Mendel, L. B., and Blood, A. F.: Some peculiarities of the proteolytic activity of papain. *J. Biol. Chem.*, **8**, 177 (1910–11).
154. Willstätter, R., Grassmann, W., and Ambros, O.: Blausäure-Aktivierung und Hemmung pflanzlicher Proteasen. *Z. physiol. Chem.*, **151**, 286 (1926). Über die Einheitlichkeit einiger Proteasen. *Ibid.*, **152**, 164 (1926).
155. Tauber, H., and Kleiner, I. S.: Some enzymes of *Solanum indicum. J. Biol. Chem.*, **105**, 679 (1934).
156. Chopra, R. N., and Roy, A. C.: Proteolytic enzyme in cucumber. *Indian J. Med. Research*, **21**, 17 (1933).
157. Maschmann, E.: Beiträge zur Aktivierung pflanzlicher Proteinasen. *Z. physiol. Chem.*, **228**, 141 (1934).
158. Schulz, F. N.: Die Verdauung der Raupe der Kleidermotte (*Tinea pellionella*). *Biochem. Z.*, **156**, 124 (1925).
159. Stankovic, R., Arnovljevic, V., and Matavulf, P.: Enzymatische Hydrolyse des Keratins mit dem Kropfsafte des *Astur palumbarius* (Habicht) und *Vultur monachus* (Kuttengeier). *Z. physiol. Chem.*, **181**, 291 (1929).
160. Mangold, E., and Dubiski: *Wiss. Arch. Landw.*, Abt. B (Tierernähr.-u. Tierzucht), **4**, 200 (1930).

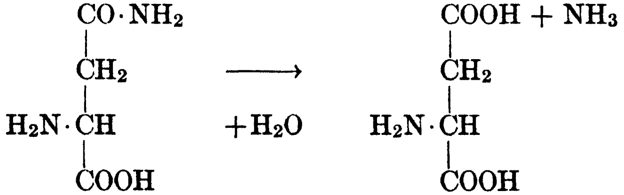
CHAPTER IV

AMIDASES

Amidases open carbon nitrogen linkages. The simplest substrates for this type of enzymes are the acid amids and amino acids. Most of these enzymes liberate ammonia from their substrates; some, however, react in a different manner (arginase, hippuricase). It has long been known that various plant and animal tissues as well as certain bacteria are good sources for amidases. Recently, however, a specific α-amino acid amidase has been obtained from the "ereptic enzymes" (1–4).

Asparaginase

Asparaginase, the enzyme which splits the amino nitrogen of asparagine, may be called absolutely specific. The product of decomposition is aspartic acid:

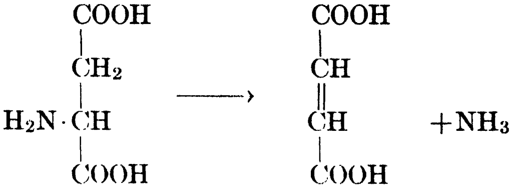


$$\begin{array}{r} \mathrm{CO\cdot NH_2} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{H_2N\cdot CH} \\ | \\ \mathrm{COOH} \end{array} \quad \xrightarrow[+\mathrm{H_2O}]{} \quad \begin{array}{r} \mathrm{COOH + NH_3} \\ | \\ \mathrm{CH_2} \\ | \\ \mathrm{H_2N\cdot CH} \\ | \\ \mathrm{COOH} \end{array}$$

The plant asparaginase of barley (5), of yeast (4, 6), and of bacteria (7) has been well studied and appears to be an individual enzyme identical with the animal asparaginase (liver) (Geddes and Hunter); (small intestine) (Grover and Chibnall). For instance, all have an optimum pH of 8 and are sensitive to acids and organic solvents. Dipeptidase, amino polypeptidase, pancreas trypsin, and pepsin do not attack asparagine (8).

ASPARTASE

Aspartase is the enzyme which is responsible for further deaminization of aspartic acid. This step yields fumaric acid:



$$\begin{array}{r}\mathrm{COOH}\\ |\\ \mathrm{CH_2}\\ |\\ \mathrm{H_2N\cdot CH}\\ |\\ \mathrm{COOH}\end{array} \longrightarrow \begin{array}{c}\mathrm{COOH}\\ |\\ \mathrm{CH}\\ \|\\ \mathrm{CH}\\ |\\ \mathrm{COOH}\end{array} + \mathrm{NH_3}$$

Aspartase may be prepared from *Bacterium coli* (7, 9). The optimum pH for both deaminization and synthesis of aspartic acid is 7.1. This enzyme is specific. No other amino acids are attacked by aspartase.

TYRAMINASE

Ewins and Laidlaw (10) were the first to observe the breakdown of tyramin to *p*-oxyphenylacetic acid. Important contributions to the chemistry of this process have been made by Chodat and Schweizer (11), by Bach (12), and more recently by Hare–Bernheim (13, 14), who studied the tyraminase of the liver. Old extracts in acid medium yield from tyramin *p*-oxyphenylacetic acid with the uptake of two atoms of oxygen. With fresh extracts, however, four atoms of oxygen combine with the tyramine, probably forming *o*-chinoidic acid. In alkaline medium, with fresh extracts, there is less oxidation. Unchanged tyramine combines with the aldehyde which forms an intermediate compound of Schiff's base.

The chemistry of enzymic deaminization is still in flux. Not many studies conducted by modern methods are available.

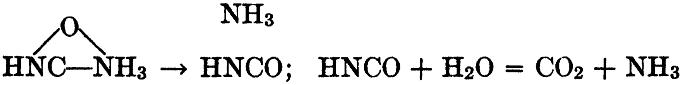
UREASE

The ammoniacal fermentation of urine, the change of urea to ammonium carbonate by *Micrococcus ureae*, has been known a long time. Later, urease was prepared from various bacteria (15–17) and from mushrooms (18, 19).

Urease is found in varying amounts in all leguminous plants. Jack bean (*Canavalia ensiformis*) and soy bean (*Glycina hispida*) are the best sources. It has been found in small quantities in the stomach (20, 21), liver (22), and other organs. No special function can be attributed to its presence in organs of higher animals.

The Intermediate Product of Urease Action

There are several theories concerning the intermediate product of urease action upon urea (23). Fearon believes that urea exists in solution as the cyclic form and the urease removes ammonia from the urea molecule with the liberation of cyanic acid, and cyanic acid would further split to carbon dioxide and ammonia.



$$\mathrm{H\overset{O}{NC}{-}NH_3 \xrightarrow{NH_3} HNCO;\quad HNCO + H_2O = CO_2 + NH_3}$$

This theory of Fearon, however, is contrary to the earlier findings of Fenton (24) and Yamasaki (25), that ammonium carbamate is the only intermediate product of urease action

$$H_2N\cdot CO\cdot NH_2 + H_2O\cdot \rightarrow H_2N\cdot CO_2\cdot NH_4$$

$$H_2N\cdot CO_2NH_4 \rightarrow CO_3(NH_4)_2$$

Later, Fearon (26) reported that he had been able to detect cyanic acid in ureolytic digests. More recently, however, Sumner, Hand, and Halloway (27) have developed a method by which the formation of ammonium carbamate and its transformation into ammonium carbonate can be clearly followed. These authors conclude that no cyanate forms. Carbamate is an intermediate product but not necessarily the first.

Specificity of Urease

Urease is absolutely specific. Methyl urea, thiourea, guanidin, and related compounds are not attacked (28, 29), nor do they show an affinity to urease (30). An exception is the hydrolysis of *n*-butyl urea by urease. This has been reported by Fearon and corroborated by Luck and Seth (28).

Preparation of Crystalline Urease

In 1926, Sumner (31) discovered how to obtain from the jack bean meal microscopic protein crystals which had very high ureolytic activity. This was the first time that an enzyme had been crystallized. The method is rather simple. One hundred grams of jack bean meal is stirred from six to eight minutes, with 500 cc. of 31.6 per cent acetone, and allowed to filter over night in a refrigerator. The octahedric crystals (see Fig. 18) which separate contain about 60 per cent of the urease activity of the filtrate. This is 25 per cent of the total urease in the 100 grams of jack bean meal.

The crystals may be recrystallized (32), and they are 700 to 1400 times more active than the jack bean meal. The crystals have an activity of about 100,000 units per gram. (A unit is the amount of urease which will form urea, 1 mg. of ammonia nitrogen in five minutes at 20° at *p*H 7.0.) Sumner's results on the preparation and activity of crystalline urease were confirmed in 1930 by the author (33), who, in collaboration with others, had prepared crystalline urease a number of times during the past eight years. At no time could a so-called inferior jack bean meal be encountered. Urease crystals were obtained without difficulty from any shipment of meal (Arlington Chemical Company, Yonkers, N. Y.).

Recently Waldschmidt-Leitz and Steigerwaldt (34) obtained a urease preparation by the adsorption method. This preparation had been concentrated 180 times and is about one-quarter as active as crystalline urease (25,000 units per gram).

The Chemical Nature of Urease

Crystalline urease is water soluble. It gives all the protein tests and the test for unoxidized sulfur. Its elementary composition is that of a protein. The isoelectric point is approximately at *p*H 5 (35). Ultra-violet irradiation inactivates urease, probably owing to a molecular rearrangement of the protein molecule (33). Urease can be inactivated, i.e., digested, by proteolytic enzymes

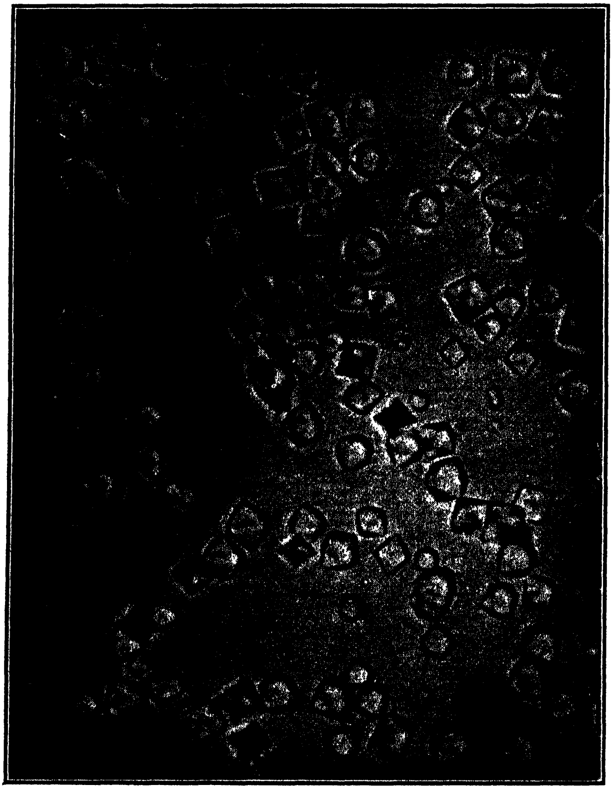


FIG. 18.—Crystalline urease. (Magnified 728 diameters)

(36, 37). This had first been noticed by Tauber (33). By digesting urease with trypsin, however, certain definite conditions are necessary (36).

More recently, Grabar and Riegert (38) have also shown that urease is a protein and that trypsin progressively inactivates it till the smallest products of digestion are inactive. Pepsin digests urease very rapidly at *p*H 4.3. Figure 19 shows the digestion of urease by pepsin (37).

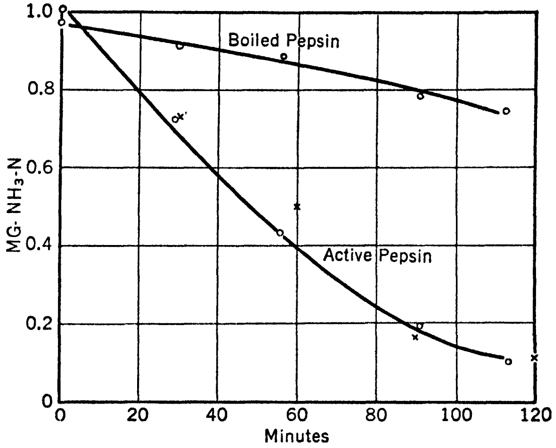


FIG. 19.—Urease digested by pepsin at 38° and *p*H 4.3. × indicates relative turbidity with dinitrosalicylic acid

Activators and Inhibitors of Urease

According to Schmidt (39), heavy metals inactivate urease in the following order: Ag, Hg, Cu, Zn, Cd, U, Au, Pb, Co, Ni, Ce, Mn. The inactivation increases with increasing *p*H (40). The activation of Ca and Ba ions in the presence of phosphate may be due to a removal of heavy metals by adsorption (41, 42).

Crystalline urease is extremely sensitive to traces of heavy metals. One gram-atom of silver inactivates more than 40,000 grams of urease (43).

Fe and I ions, as well as free I, inhibit (44). Alcohol does not inhibit, even in high concentrations (45). That the sulfhydryl radical is a part of the catalytic function of the urease molecule has been recently shown by Sumner and associates (46). Hellerman, Perkins, and Clark (47) verified this contention by supplementing it with very interesting and convincing experiments, indicating that heavy metal inactivation of urease is based on the oxidation of the SH group and that this reaction is reversible; i.e., by changing the S–S group again to the S–H group (by treatment with H_2S or other sulfhydryl compounds) practically all the ureolytic activity may be regained.

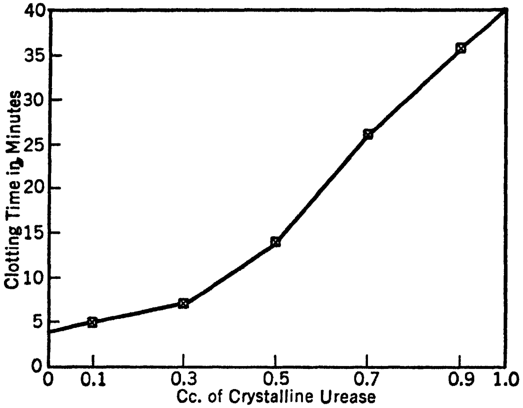


FIG. 20.—Inhibition of rennet activity by crystalline urease. Increasing the amount of inhibitor increases the milk-clotting time. The aqueous solution of urease was at *p*H 6.1. For the determination of milk-clotting power milk of *p*H 6.3 was used. The temperature was 37°

Quastel has found that all basic dyes inhibit urease action but not the acidic dyes (48).

Urease does not require an activator or a coenzyme (49, 49*a*).

Solutions of crystalline urease are powerful chymoinhibitors (50). They inhibit the milk-clotting power of rennin, pepsin, and trypsin. Rennin, however, is much more inhibited than pepsin and trypsin. A typical experiment is represented in Fig. 20. Blood serum has a similar property, which is probably due to a combination of protein with the active group of the enzyme.

Reaction Course of Urease

Earlier investigations have disregarded the factors influencing urease activity. In solutions which are not sufficiently buffered, urease is destroyed by the rapid alkalinization of the digest, owing to ammonium carbonate formation. Van Slyke and Cullen (51) have shown that, when the initial concentration of the urea is increased, there is a gradual increase in hydrolysis at equal periods, until a maximum is reached. This is true when the *p*H is constant and the urea solutions are diluted. The dissociation constant of the urease compound is about 0.025. The principle of the reaction course is not clear. Van Slyke and Cullen divided the urea-urease reaction into two processes having different reaction velocities: (*a*) The formation, and (*b*) the splitting, of the urea-urease compound. The reaction velocity is ascribed to the reaction velocity of the sum of a linear and logarithmic function. The experiments of Barendrecht (52) and of Lövgren (53) did not confirm this. Lövgren has found that the reaction course for the initial stage follows an equation of the first order.

Physiological Properties of Urease

The toxicity of urease when administered intravenously or subcutaneously into the animal body was attributed to various causes. The results of Tauber and Kleiner (54), who have also studied the toxicity of ammonium carbonate, point directly to ammonia poisoning. An immunity to urease, however, was acquired by the experimental animal with the injection of increasing amounts, starting below the lethal dose. Kirk and Sumner (55) described a method for the preparation of antiurease. Howell (56) found that the hen cannot be poisoned by urease since it has only 2 mg. of urea per 100 cc. of blood; antiurease is formed, however.

The Effect of Buffers upon Urease Action

The reason for the above contradictory results concerning the reaction course of urease may be due to the fact that some of these experiments have been carried out in the presence of phosphate buffers. The earlier workers estimated the action of phosphate upon urease as well as the action of urease upon urea. Krebs and Henseleit (57) have definitely shown that, at *p*H 5, phosphate buffer inhibits urease activity.

Van Slyke and Zacharias (58) and Lövgren (59) have observed that the *p*H optimum for urease shifts to the alkaline side with decreasing concentrations of urea. Howell and Sumner (60) recently studied the effects of buffers upon urease action. They found that the shift to the alkaline side is considerable with phosphate buffer, but only slight with acetate or citrate buffer respectively. The activity of urease depends upon the type of buffer, the temperature, *p*H, urea concentration, and salt concentration (see also Chapter I).

HIPPURICASE OR HISTOZYME

The synthesis and hydrolysis of benzoylated amino acids such as hippuric acid (benzoyl glycocoll) into benzoic acid and glycocoll was attributed by Schmiedeberg (61) to an individual enzyme which he called histozyme. Neuberg found (62*a*) this enzyme to be present in the mold *Aspergillus oryzae.* The liver, pancreas, and other organs, as well as muscles of mammals, contain it also. Hippuricase splits acid radicals from peptides and amino acids of the type $R—CO \cdot NH—CHR^1—COOH$. Simple dipeptides, therefore, are not hydrolyzed. Only derivatives of natural amino acids are split (63). The enzyme may be employed in the separation of racemic compounds (64, 65). Benzoyl derivatives of β-amino acids, aspartic acid, and glutamic acid are not hydrolyzed, whereas benzoyl asparagin is hydrolyzed (66). Glycoholic acid and taurocholic acid, however, are split by hippuricase (67).

The optimum *p*H of hippuricase is at 6.8 to 7.0 and is independent of the substrate.

ARGINASE

Arginase was discovered in 1904 by Kossel and Dakin (68). It decomposes arginine (the *d*-form, not the laevo) (guanido amino valeric acid) into ornithine (diaminovaleric acid) and urea. This is an important phase of intermediary protein metabolism.

Source

The best source of arginase is the male mammal's liver (69), the male liver containing much more than the female. A specific arginine metabolism has been recognized. It is increased after sexual maturity. Contrary to earlier statements, Edlbacher (70, 71) found that there is no arginase in other organs besides the liver. Recent work of Weil (72) indicates that there are probably traces of arginase in all body fluids and tissues of the mammal.

Preparation of Crude Arginase

(*a*) *Preparation of Glycerol Suspension.* Rats are bled and the livers removed and frozen in liquid nitrogen, pulverized, and suspended in 10 parts of 90 per cent glycerol. For one estimation, 0.25 cc. of the suspension is sufficient (72).

(*b*) *Preparation of Glycerol Extract.* The liver tissue, which has been frozen in liquid nitrogen and pulverized, is extracted with several portions of acetone, acetone-ether, and finally with ether. The dry preparation is sifted, suspended in 10 parts of 90 per cent glycerol for one day, and filtered. For one estimation, 1 cc. of extract should be used (72).

Preparation of Purified Arginase

Twenty-five cubic centimeters of the glycerol extract is diluted with 100 cc. of H_2O and five times adsorbed with alumina $C\gamma$ suspension (1 cc. 35 mg. of Al_2O_3) at *p*H 6.5. The combined adsorbates are washed with H_2O and eluted four times with 12 cc. of $M/15$ Na_2HPO_4. The elution is then filtered through kieselguhr and precipitated with a double volume of acetone in an ice bath. The precipitate is now dissolved in 20 cc. of 0.1 *M* glycine NaOH buffer of *p*H 9.5 and dialyzed for three hours against running water. For one estimation, 3 cc. of the solution should be used (73). The optimum *p*H for arginase activity is 9.5 to 9.9 (Edlbacher and Bonen).

Activation and Estimation of Arginase

Prepare tissue extracts as described above. The simple method as given in (*a*) will suffice for ordinary work.

Activate tissue arginase by adding to 5 cc. glycerol suspension of *p*H 7.0, 2 cc. of cysteine hydrochloride (20 mg.), or 0.5 cc. of 0.1 *N* $FeSO_4$, or both, and incubate for one hour at 30°. Then add 10 cc. of arginine carbonate (100 mg.) and 5.0 cc. of 0.1 *M* glycine NaOH buffer of *p*H 9.5, and dilute to 25 cc. with water. Incubate for one hour at 30°. The urea formed may now be determined with urease by titration, colorimetrically (74) or manometrically (74). Weil found (see Table XIV) that, invariably, the activation is strongest when both cysteine and $FeSO_4$ are present. By using the above procedures, he determined the liver arginase content of rats suffering from cancer and noticed that those animals tend to be low in arginase.

Mechanism of Arginase Activation

The mechanism of arginase activation has been extensively studied recently. It was assumed at first that sulfhydryl compounds are specific activators of arginase (75,

TABLE XIV

ACTIVATION OF ARGINASE OBTAINED FROM VARIOUS SOURCES AND OF DIFFERENT DEGREES OF PURITY

Enzyme preparation	Amount of enzyme	Arginase activity			
		Initial	$\mathrm{Fe''}$	Cysteine	Cysteine + $\mathrm{Fe''}$
	cc.	cc.	cc.	cc.	cc.
Glycerol suspension of liver (1 : 10)	0.25	17.5	14.7	20.7	19.8
	0.25	11.2	10.4	14.1	16.1
	0.25	14.3	10.7	20.4	22.2
	0.25	11.3	12.8	16.5	24.5
Acetone-ether-glycerol extract	1.0	11.3	22.8	7.0	23.4
	1.0	6.7	14.2	7.0	23.1
	1.0	7.6	19.9	11.0	21.0
	1.0	10.0	18.2	12.1	19.1
Purified enzyme	3.0	6.2	17.8	8.1	19.3
Glycerol suspension of tumor tissue (transplanted rat sarcoma)	5.0	3.5	10.8	4.2	10.1
	5.0	2.9	7.1	4.9	7.7
	5.0	0.2	6.6	1.0	8.6
	5.0	0.6	4.8	2.6	4.5
	5.0	1.9	5.5	1.7	5.9

76). This view was not accepted, since it has been found that the effect of the sulfhydryl group depended on the purity of the arginase as well as the *p*H of the solution (77, 78). Later, it was noticed that sulfhydryl compounds combined with heavy metal ($\mathrm{Fe''}$ or $\mathrm{Cu'}$) function as specific activators of this enzyme (79, 80). Purr and Weil (81), however, have shown that sulfhydryl compounds cannot be classified as specific activators, since other products of intermediary metabolism (alloxan-$\mathrm{Fe''}$, ascorbic acid-$\mathrm{Fe''}$, and methylglyoxal-$\mathrm{Fe''}$), combined with iron, can activate arginase. The findings of Klein and Ziese (82) are similar. These investigators also found that oxidizing agents had an activating effect on purified arginase but not on crude preparations. The action is based on simple oxidation. It is not dependent on the formation of a definite oxidation-reduction potential.

Edlbacher, Kraus, and Leuthardt (83), however, find oxygen inhibitive, the cysteine-Fe″ complex acting solely as a protection against oxygen. In a recent paper, Leuthardt and Köller (84) state that sulfhydryl compounds, besides acting as a protection against oxygen, are capable of exerting another yet unknown effect.

Weil (85) has shown that, regardless of whether an enzyme preparation is purified or crude, the cysteine-Fe″ complex is capable of activating the arginase. On the other hand, it is a fact that the mechanism of arginase activation is not known. Further experiments will have to be carried out to elucidate the chemistry of this important factor.

Purine Amidases

Purine amidases are present in mammal's liver and muscles respectively. Their function is to deaminize purines.

The early investigations of Minkowski and Jones (1904) have been continued by Schmidt (86–88), who has been able to furnish a series of very interesting experiments concerning purine amidases.

Guanine is converted to xanthine by guanase, the enzyme of the rabbit's liver, but not by muscle extracts. The muscle extracts attack adenosin (adeninribosid) as well as adenylic acid (adenosinphosphoric acid) but not guanine, guanosine, and guanylic acid. The deaminization of adenylic acid and adenosin is carried out by two different enzymes, adenylic acid deaminase and adenosin deaminase. The former is soluble in bicarbonate solutions; the latter is not. Thus, a separation of the two is possible. Adenosin deaminase may be obtained free of adenylic acid deaminase by extraction of muscle tissue with slightly acidified salt solutions. Adenylic acid deaminase has an optimum *p*H of 5.9. It does not split amino acids, uric acid, creatine, creatinine, adenine, adenosine, or guanine and its derivatives. Neither does it split the isomer of muscle adenylic acid, namely, yeast adenylic acid.

The enzyme complex of the liver (rabbit) is much more complicated. It breaks down adenosine, adenylic acid, guanine, guanosine, and guanylic acid, as well as cytosine and cytosylic acid. It also contains nucleophosphatases. Elutions obtained from alumina adsorbates hydrolyze rapidly guanine and the nucleosides of adenine and guanine but not the phosphoric acid ester of guanine. They are free of phosphatase.

The adenosin-deaminizing enzyme may be obtained free from guanase by selective elution. This guanine-deaminase or guanase has an optimum *p*H of 9.2, whereas the guanylic acid deaminase has an optimum of 5.3. Guanase does not break down arginine, creatine, creatinine, or related compounds.

REFERENCES

1. Grassmann, W., and Dyckerhoff, H.: Über die Spezifität der Hefe-Peptidasen (Zweite Abhandlung über Pflanzen-Proteasen in der von R. Willstätter und Mitarbeiter begonnenen Untersuchungsreihe). *Ber.*, **61**, 656 (1928).
2. Waldschmidt-Leitz, E., and Balls, A. K.: Zur Kenntnis der Amino-Polypeptidase aus Darm-Schleimhaut (Neunzehnte Mitteilung zur Spezifität tierischer Proteasen). *Ber.*, **63**, 1203 (1930).
3. Linderstroem-Lang, K.: Studies on proteolytic enzymes. 9. On the cleavage of leucyldecarboxyglycine by intestinal erepsin. *Compt. rend. trav. lab. Carlsberg*, **19**, 3 (1931).
4. Grassmann, W., and Mayr, O.: Zur Kenntnis der Hefeasparaginase. *Z. physiol. Chem.*, **214**, 185 (1933).
5. Grover, C. E., and Chibnall, A. C.: The enzymic deamidation of asparagine in the higher plants. *Biochem. J.*, **21**, 857 (1927).
6. Geddes, W. F., and Hunter, A.: Observations upon the enzyme asparaginase. *J. Biol. Chem.*, **77**, 197 (1928).
7. Virtanen, A., and Tarnanen, J.: Die enzymatische Spaltung und Synthese der Asparaginsäure. *Biochem. Z.*, **250**, 193 (1932).

8. Damodaran, M.: The isolation of asparagine from an enzymic digest of edestin. *Biochem. J.*, **26**, 235; The isolation of glutamine from an enzymic digest of gliadin. *Biochem. J.*, **26**, 1704 (1932).
9. Quastel, J. H., and Woolf, B.: The equilibrium between *l*-aspartic acid, fumaric acid and ammonia in presence of resting bacteria. *Biochem. J.*, **20**, 545 (1926).
10. Ewins, A. J., and Laidlaw, P.: The fate of parahydroxyphenylethylamine in the organism. *J. Physiol.*, **41**, 78 (1910–11).
11. Chodat, R., and Schweizer, K.: Über die desamidierende Wirkung der Tyrosinase. *Biochem. Z.*, **57**, 430 (1913).
12. Bach, A.: Über das Wesen der sogenannten Tyrosinasewirkung. *Biochem. Z.*, **60**, 221 (1914).
13. Hare, M. L. C.: Tyramine oxidase. *Biochem. J.*, **22**, 968 (1928).
14. Bernheim, M. L. C.: Tyramine oxidase. II. The cause of the oxidation. *J. Biol. Chem.*, **93**, 299 (1931).
15. Jacoby, M.: Über eine einfache und sichere Methode der Ureasedarstellung aus Bakterien. *Biochem. Z.*, **84**, 354 (1917).
16. Takahata, T.: Preparation of a urease solution from bacteria. *J. Chem. Soc.*, Abstr., **124**, Part I, 1157 (1923).
17. Iwanoff, N. N., and Smirnowa, M. I.: Über Harnstoff bei Bakterien. II. *Biochem. Z.*, **181**, 8 (1927).
18. Sumi, M.: Über die chemischen Bestandteile der Sporen von *Aspergillus oryzae*. *Biochem. Z.*, **195**, 161 (1928).
19. Bach, M. D.: Évolution de l'uréase dans les cultures de *l'Aspergillus niger*. *Bull. soc. chim. biol.*, **11**, 1007; L'uréase et l'asparaginase de *l'Aspergillus niger* sont-elles des endodiastases? *Ibid.*, **11**, 1016 (1929).
20. Rigoni, M.: Ricerche sull'ureasi: III. L'ureasi nella mucosa gastrica dell'uomo e degli animali. *Arch. sci. biol. Italy*, **15**, 37 (1930).
21. Majorow, S.: Über das Vorhandensein von Urease in Organismus der Tiere. *Biochem. Z.*, **241**, 228 (1931).
22. Steppuhn, O., and Utkin-Ljubowzoff, X.: Versuche zur Erfassung einer tierischen Urease. *Biochem. Z.*, **146**, 115 (1924).
23. Fearon, W. R.: Urease. Part I. The chemical changes involved in the zymolysis of urea. *Biochem. J.*, **17**, 84. Part II. The mechanism of the zymolysis of urea. *Ibid.*, **17**, 800 (1923).
24. Fenton, H. J. H.: On the limited hydration of ammonium carbamate. *Proc. Roy. Soc. London*, **39**, 386 (1885).
25. Yamasaki, E.: Tohokee Imperial Univ. Sc. Rep. Series I, **9**, 96 (1920).
26. Fearon, W. R.: The significance of cyanic acid in the urea-urease system. *J. Biol. Chem.*, **70**, 785 (1926).
27. Sumner, J. B., Hand, D. B., and Halloway, R. G.: Studies of the

intermediate products formed during the hydrolysis of urea by urease. *J. Biol. Chem.*, **91**, 333 (1931).

28. Luck, J. M., and Seth, T. N.: Ammonia production by animal urease in vitro. *Biochem. J.*, **18**, 825 (1924).
29. Münch, H.: Beitrag zum Mechanismus der Ureaseaktivierung. *Z. physiol. Chem.*, **187**, 241 (1930).
30. Ambros, O., and Münch, H.: Studien zum Mechanismus der Ureasewirkung. *Z. physiol. Chem.*, **187**, 252 (1930).
31. Sumner, J. B.: The isolation and crystallization of the enzyme urease. *J. Biol. Chem.*, **69**, 435 (1926).
32. Sumner, J. B.: The recrystallization of urease. *J. Biol. Chem.*, **70**, 97 (1926).
33. Tauber, H.: Studies on crystalline urease. Inactivation by ultraviolet radiation, sunlight with the aid of a photodynamic agent, and inactivation by trypsin. *J. Biol. Chem.*, **87**, 625 (1930).
34. Waldschmidt-Leitz, E., and Steigerwaldt, F.: Über die chemische Natur der Urease. *Z. physiol. Chem.*, **195**, 260 (1931).
35. Sumner, J. B., and Hand, D. B.: The isoelectric point of crystalline urease. *J. Am. Chem. Soc.*, **51**, 1255 (1929).
36. Tauber, H., and Kleiner, I. S.: Studies on crystalline urease. IV. The "antitryptic" property of crystalline urease. *J. Gen. Physiol.*, **15**, 155 (1931–32).
37. Sumner, J. B., Kirk, J. S., and Howell, S. F.: The digestion and inactivation of crystalline urease by pepsin and by papain. *J. Biol. Chem.*, **98**, 543 (1932).
38. Grabar, P., and Riegert, A.: *Compt. rend.*, **200**, 1795 (1935).
39. Schmidt, E. G.: The inactivation of urease. *J. Biol. Chem.*, **78**, 53 (1928).
40. Kitagawa, M.: On the influence of hydrogen-ion concentration upon the inactivation of urease by some heavy metal salts. *J. Biochem.*, **10**, 197 (1928–29).
41. Hosokawa. T.: Über Auxoureasen. *Biochem. Z.*, **149**, 363 (1924).
42. Kochmann, R.: Über Auxoureasen, der Mechanismus der Kalkwirkung. *Biochem. Z.*, **151**, 259 (1924).
43. Sumner, J. B., and Myrbäck, K.: Über Schwermetall-Inaktivierung hochgereinigter Urease. *Z. physiol. Chem.*, **189**, 218 (1930).
44. Jacoby, M.: Über die Einwirkung des Fluors und des Jods auf die Urease. *Biochem. Z.*, **214**, 368 (1929).
45. Rosenfeld, L.: Über das Verhalten der Urease gegen Alkohol. *Biochem. Z.*, **154**, 141 (1924).
46. Sumner, J. B., and Poland, L. O.: Sulfhydryl compound and crystalline urease. *Proc. Soc. Exptl. Biol. Med.*, **30**, 553 (1932–33).
47. Hellerman, L., Perkins, M. E., and Clark, W. M.: Urease activ-

ity as influenced by oxidation and reduction. *Proc. Nat. Acad. Sci.*, **19**, 855 (1933).

48. QUASTEL, J. H.: The action of dyestuffs on enzymes. III. Urease. *Biochem. J.*, **26**, 1685 (1932).
49. LOEB, L., and LORBERBLATT, I.: Über die Spezifität der in den Amoebocyten von Limulus enthaltenen Urease mit Versuchen über das Verhalten von erwärmten und dialysierten Extrakten von Amoebocytengewebe. *Biochem. Z.*, **244**, 222 (1932).

49*a*. SUMNER, J. B., and KIRK, J. S.: Is there a co-enzyme for urease? *Biochem. J.*, **26**, 551 (1932).

50. TAUBER, H.: Inhibitors of milk-curdling enzymes. *J. Biol. Chem.*, **107**, 161 (1934).
51. VAN SLYKE, D. D., and Cullen, G. E.: The mode of action of urease and of enzymes in general. *J. Biol. Chem.*, **19**, 141 (1914).
52. BARENDRECHT, H. P.: L'uréase et la théorie de l'action des enzymes par rayonnement. *Rec. trav. chim.*, **39**, 2 (1920).
53. LÖVGREN, S.: Studien über die Urease. *Biochem. Z.*, **119**, 215 (1921).
54. TAUBER, H., and KLEINER, I. S.: Studies on crystalline urease. III. The toxicity of crystalline urease. *J. Biol. Chem.*, **92**, 177 (1931).
55. KIRK, J. S., and SUMNER, J. B.: Antiurease. *J. Biol. Chem.*, **94**, 21 (1931).
56. HOWELL, S. F.: Antiurease formation in the hen. *Proc. Soc. Exptl. Biol. Med.*, **29**, 759 (1932).
57. KREBS, H. A., and HENSELEIT, K.: Untersuchungen über die Harnstoffbildung im Tierkörper. *Z. physiol. Chem.*, **210**, 33 (1932).
58. VAN SLYKE, D. D., and ZACHARIAS, G.: The effect of hydrogen-ion concentration and of inhibiting substances on urease. *J. Biol. Chem.*, **19**, 181 (1914).
59. LÖVGREN, S.: Studien über die Urease. II. *Biochem. Z.*, **137**, 206 (1923).
60. HOWELL, S. F., and SUMNER, J. B.: The specific effects of buffers upon urease activity. *J. Biol. Chem.*, **104**, 619 (1934).
61. SCHMIEDEBERG, O.: Über Oxydationen und Synthesen im Thierkörper. *Arch. exptl. Path. Pharmakol.*, **14**, 288 (1881). Über Spaltungen und Synthesen im Thierkörper. *Ibid.*, 379.
62. NEUBERG, C., and ROSENTHAL, O.: Über Taka-Lactase. *Biochem. Z.*, **145**, 186 (1924).

62*a*. NEUBERG, C., and NOGOUCHI, J.: Über die enzymatische Spaltung der Phenacetursäure. *Biochem. Z.*, **147**, 370 (1924).

63. SMORODINZEW, A.: Über die Wirkung des Histozyms auf die Homologen der Hippursäure. *Z. physiol. Chem.*, **124**, 123 (1922–23).
64. NEUBERG, C., and LINHARDT, K.: Die enzymatische Spaltung benzoylierter Aminosäuren und ihr asymmetrischer Verlauf. *Biochem. Z.*, **147**, 372 (1924).

65. Hoppert, C.: Über ein neues biochemisches Verfahren zur Spaltung razemischer Aminosäuren. *Biochem. Z.*, **149**, 510 (1924).

66. Takehiko, S.: Über die Hydrolyse der Benzoylderivate der Aminosäuren durch das Histozym. *J. Biochem.*, **12**, 107 (1930).

67. Grassmann, W., and Basu, K. P.: Über die enzymatische Spaltbarkeit gepaarter Gallensäuren. *Z. physiol. Chem.*, **198**, 247 (1931).

68. Kossel, A., and Dakin, H. D.: Über die Arginase. *Z. phsyiol. Chem.*, **41**, 321 (1904).

69. Fuchs, B.: Über das Vorkommen der Arginase in gesunden und kranken Organismus. *Z. physiol. Chem.*, **114**, 101 (1931).

70. Edlbacher, S.: Versuche über Wirkung und Vorkommen der Arginase. *Z. physiol. Chem.*, **100**, 111 (1917).

71. Edlbacher, S., and Bonem, P.: Beiträge zur Kenntnis der Arginase. *Z. physiol. Chem.*, **145**, 69 (1925).

72. Weil, L.: The action of arginase. *J. Biol. Chem.*, **110**, 201 (1935).

73. Waldschmidt-Leitz, E., Scharikova, A., and Schäffner, A.: Über den Einfluss von Sulfhydrylverbindungen auf enzymatische Prozesse. *Z. physiol. Chem.*, **214**, 75 (1933).

74. Weil, L., and Russell, M. A.: Manometric micromethod for arginase determination; enzymatic study of blood arginase in rats. *J. Biol. Chem.*, **106**, 505 (1934).

75. Salaskin, S., and Solowjew, L.: Über Beeinflussung der Arginase durch Säuerstoff, Kohlensäure und Cystein. *Z. physiol. Chem.*, **200**, 259 (1931).

76. Waldschmidt-Leitz, E., Schäffner, A., and Kocholaty, W.: Über die Bedeutung des Glutathione für den Stoffwechsel: *Naturwissenschaften*, **19**, 964 (1931).

77. Edlbacher, S., Kraus, J., and Walter, G.: Beiträge zur Kenntnis der Arginase (Siebente Mitteilung. Aktivierungs und Hemmungsversuche). *Z. physiol. Chem.*, **206**, 65 (1932).

78. Klein, G., and Ziese, W.: Studien über Tumorarginase. I. Zur Frage der Aktivierbarkeit von Leberarginase durch Cystein und Glutathion. *Z. physiol. Chem.*, **211**, 23 (1932).

79. Salaskin, S., and Solowjew, L.: Aktivierbarkeit und Hemmung auf verschiedene Weise hergestellter Arginase durch Sauerstoff, Kohlensäure, Cystein und Schwarmetallsalze. II. *Biochem. Z.*, **250**, 503 (1932).

80. Waldschmidt-Leitz, E., Scharikova, A., and Schäffner, A.: Über den Einfluss von Sulfhydrylverbindungen auf enzymatische Prozesse. *Z. physiol. Chem.*, **214**, 75 (1933).

81. Purr, A., and Weil, L.: The relation of intermediary metabolic products to arginase activation. *Biochem. J.*, **28**, 740 (1934).

82. Klein, G., and Ziese, W.: Das Verhalten von Oxydationsmitteln gegenüber gereinigter Arginase. *Z. physiol. Chem.*, **229**, 209 (1934).

83. Edlbacher, S., Kraus, J., and Leuthardt, F.: Die Steuerung der Arginasewirkung durch Sauerstoff (Neunte Mitteilung zur Kenntnis der Arginase). *Z. physiol. Chem.*, **217**, 89 (1933).
84. Leuthardt, F., and Köller, F.: Über die Aktivatoren der Arginase. *Helv. Chim. Acta*, **17**, 1030 (1934).
85. Weil, L.: The activation of arginase. *J. Biol. Chem.*, **110**, 201 (1935).
86. Schmidt, G.: Über fermentative Desaminierung in Muskel. *Z. physiol. Chem.*, **179**, 243 (1928).
87. Schmidt, G.: Über den ferementativen Abbau der Guanylsäure in der Kaninchenleber. *Z. physiol. Chem.*, **208**, 185 (1932).
88. Schmidt, G.: Über den Abbau des Guaninkerns durch die Fermente der Kaninchenleber. *Klin. Wochenschr.*, **10**, 165 (1931).

CHAPTER V

CARBOHYDRASES

SUCRASE (SACCHARASE, INVERTASE)

Sucrase occurs in the small intestine of mammals and in the tissues of certain animals and plants. Sucrase hydrolyzes cane sugar into fructose and glucose. It may be obtained in a relatively pure state from yeast, which is a very good source. Sucrase is quite a stable enzyme. Cane sugar, the substrate of sucrase, is inexpensive. The end products of hydrolysis are easily determined. For these reasons, the chemistry of sucrase action has been extensively studied by many chemists.

Preparation of Sucrase. Various procedures have been tried. Emil Fischer (1, 1*a*) extracted yeast dried at room temperature with water to which some toluene had been added. Kjeldahl (2) and Michaelis (3) extracted yeast, which had been ground with sand, with water containing some chloroform.

O'Sullivan and Tompson (4) introduced a method which is still much in use. They allowed the yeast to autolyze at room temperature until the yeast cells died and partially liquefied. Hudson (5) speeded up autolysis by the addition of toluene, so that within a few days liquefaction was complete. He added lead acetate to remove soluble proteins and gums. The excess lead was removed by the addition of potassium oxalate, and the excess of the potassium oxalate by dialysis. The crude sucrase was then precipitated by an equal volume of alcohol. The resulting preparation was dissolved in water.

Von Euler (6, 7) and associates omitted the lead treatment, stating that a preparation of similar activity may be obtained by adding alcohol to the autolyzed fluid and extracting the resulting precipitate with water. To the aqueous extract they then added alcohol and extracted the second precipitate with water. Willstätter (8, 9) and coworkers obtained highly active preparations by modifying the Hudson method. They interrupted autolysis about three hours after toluene or chloroform addition and filtered the liquid. The filtrate was discarded, since it contained only a trace of the enzyme. Then toluene was added to the residue and allowed to autolyze completely. Willstätter and Racke (10) suggested autolysis in neutral instead of acid medium, so as to remove more protein matter. They also studied the purification of invertase by the adsorption method and found that an acid *p*H was most suitable (10*a*). The principle of this method was as follows: Impurities were first removed by dialysis of the autolysate, or by the application of the lead-alcohol precipitation method as suggested by Hudson. After the preliminary treatment, the sucrase was adsorbed to kaolin from the acidified solution and then eluted by the addition of diluted ammonia, sodium carbonate, or phosphate. The solution was then dialyzed and adjusted to *p*H 5. The invertase was adsorbed by alumina and eluted by means of disodium phosphate or arsenate. The resulting solution was dialyzed. By repeating the alumina procedure, very active preparations were obtained. The kaolin removes the gums, and the alumina removes nitrogenous matter.

Lutz and Nelson (11) recently obtained a highly active sucrase from yeast. They found the adsorption-elution method of Willstätter and associates (references 12 and 13) inadequate for large-scale operations and devised a method which is based to a certain extent on several earlier procedures (autolysis, precipitation by alcohol, kaolin adsorption, elution from kaolin with secondary ammonium phosphate, dialysis of the eluate, adsorption of this on alumina, elution with secondary sodium phosphate, ammonium sulfate treatment, dialysis). This sucrase was free of protein. It has been found, since the paper of Lutz and Nelson (11) appeared, that the dry enzyme preparation and its aqueous solutions show good protein and yeast gum tests (13*a*).

Optimum *p*H. The optimum *p*H for yeast sucrase activity was found to be 4.5 (14).

The kinetics of sucrase activity has been excellently discussed by Nelson (15) and by Weidenhagen (16). Both reviewers emphasize the important influence of the Michaelis-Menten theory concerning enzyme specificity (see also Chapter I).

Methods for the Estimation of Sucrase Activity. The time necessary to bring the rotation of a sucrose solution to 0 is estimated with the aid of the polariscope, and is called the " time value." Thus, according to Willstätter (10, 10*a*, 17), the enzyme preparation has the potency of 1 sucrase unit when 50 mg. have a time value of 1, when 25 cc. of 16 per cent sucrose at 15.5° is used. This means that the purity of a sucrase preparation is expressed by the enzyme value, or number of units in 50 mg. of enzyme preparation. The unit of Euler and coworkers (18, 19) is the *Inversionsfähigkeit* (power of inversion) where $If = (k \times \text{grams of substrate}) \div \text{grams of enzyme}$. Sumner and Howell (20, 21) propose the dinitrosalicylic acid method for reducing sugars for the estimation of sucrase activity, which would be much faster and simpler than the earlier methods. They use sucrose concentrations (5 to 10 per cent) so as to obtain maximum velocity of sucrase activity (22). In the digest of Sumner and Howell, no more sucrase is used than is necessary to obtain 10 mg. of invert sugar by hydrolysis of 6 cc. of 5.4 per cent sucrose in 5 minutes at 20°. Under these conditions, they found that the velocity is only 1 per cent less than it is at 0 time. The reaction is stopped by the addition of 5 cc., approximately 0.1 N NaOH, and the invert sugar is determined colorimetrically. By this method, the sucrose units are expressed in terms of milligrams of invert sugar produced in 5 minutes at 20°, at *p*H 4.5 (*N* acetate buffer). Probably other colorimetric methods can also be used.

The Specificity of Hexosidases. The theory of Leibowitz (1925) states that there are two kinds of maltases, one which hydrolyzes maltose and α-methylglucoside, and another which splits only maltose. The latter is present in certain molds; the former in yeast. Fischer and Niebel (23) found, as early as 1896, that the maltase of horse serum would attack only maltose and not α-methylglucoside.

Weidenhagen (24, 24*a*), however, denies the existence of a specific sucrase, maltase, or α-methylglucosidase. He has proposed a theory according to which sucrose is hydrolyzed by α-*n*-glucosidase because it is an α-*n*-glucoside and by β-*h*-fructosidase because it is also a β-*h*-fructoside; maltose, on the other hand, being only an α-*n*-glucoside, is split only by α-*n*-glucosidase. Weidenhagen extends the same opinion to other saccharides. Much interest has been aroused in this new theory, but it has not found general acceptance.

For example, Karstroem (25), Myrbäck (26), and Tauber and Kleiner (27), using enzyme preparations of certain strains of *Bacterium coli*, found that maltose could be hydrolyzed but not sucrose. Pringsheim, Borhardt, and Loew (28) found malt extracts and certain molds to be inactive toward α-methylglucoside but not toward maltose. Iwanoff, Dodonowa, and Tschastuchin (29) obtained from mushrooms a maltase which was inert to sucrose but not to maltose. Schubert (15), working in Nelson's laboratory, found a sucrase which did not hydrolyze α-methylglucoside.

A maltase inert to sucrose and α-methylglucoside has been obtained by Kleiner and Tauber (30) from mammary tissue. Grassmann (31, 32) and associates, and recently Hotchkiss (33), by using a great number of bacterial preparations, have shown that the theory of Weidenhagen is invalid. More recently, Tauber and Kleiner (34) have tested the theory of Weidenhagen again. Since Weidenhagen has stated that α-phenylglucoside is more easily hydrolyzed by maltase than α-methylglucoside, Tauber and Kleiner employed the new substrate. They used the maltase obtained from *Solanum indicum.* It did not hydrolyze α-methylglucoside. It did, however, slowly split α-phenylglucoside, and maltose was rapidly hydrolyzed. Therefore, it was suggested that the maltases be divided into two groups. The first, or *true α-glucosidases,* split all α-glucosides and maltose, e.g., yeast maltase. The second or *pseudo α-glucosidases* hydrolyze maltose; they are only slightly active to certain α-glucosides, and are inert to all others. To the second group belong maltase of *B. coli,* maltase of mammary gland, malt extracts, and the maltase of *Solanum indicum.* This new classification explains earlier discrepancies.

Regarding the specificity of sucrases, there is no general agreement. According to Kuhn and associates (35) there are two types of sucrases. Some taka-saccharases do not hydrolyze raffinose; others do. Leibowitz and Mechlinski (36) repeated these experiments and found that some taka-saccharase preparations contain melibiase, which is responsible for the splitting of raffinose, and others do not. For instance, the trisaccharide was split by the melibiase to galactose and sucrose, and the sucrose freed in this manner was then hydrolyzed by the taka-saccharase. This was contradicted by Weidenhagen (16). He found that taka-saccharase preparations, containing no melibiase, could attack raffinose. Tauber and Kleiner (34) obtained an enzyme preparation from *Solanum indicum* which readily split raffinose, but the enzymes melibiase and sucrase were also present.

α-*d*-GLUCOSIDASES: MALTASES

Maltases are found in almost all plant and animal tissues. Yeast is a good source of maltase. The substrates for these enzymes are maltose and α-glucosides. Their optimum *p*H is close to 7.0, varying slightly according to the source and buffers employed.

It has been stated (Specificity of Hexosidases) that there are two classes of maltases: (*a*) true α-glucosidases and (*b*) pseudo α-*d*-glucosidases. The first group splits maltose and all α-glucosides. The second group hydrolyzes maltose and those α-*d*-glucosides which are easily split.

Purification of Yeast Maltase and Its Separation from Sucrase. Michaelis and Rona (37) were able to separate yeast maltase and sucrase by treating yeast autolysate with kaolin. The kaolin adsorbed the maltase whereas the sucrase remained in solution. Willstätter and Bamann (38) confirmed this, but suggested that certain hydroxides of aluminum are better adsorbents since they are not as destructive to the maltase as is the kaolin. The methods for the preparation of those gels and experiments showing selective adsorption are described by Willstätter, Kraut, and Erbacher (39).

Kinetics. The kinetics of maltose hydrolysis varies with each yeast and with each substrate. The reaction proceeds more slowly than the monomolecular one. This has been shown by several earlier workers and has been corroborated by Willstätter, Oppenheimer, and Steibelt (40, 40*a*).

Synthetic Action of Maltase (or True α-Glucosidase). Aubry (41) and Bourquelot (42) described a simple method for the synthesis of α-methylglucoside from methyl alcohol and glucose. The method is as follows: Place in a 10-liter flask 1800 grams of pure methyl alcohol and 500 grams of glucose dissolved in 4 liters of distilled water. Mix. Add 3 liters of filtered yeast macerate (to contain 10 per cent dry bottom yeast). Mix. Dilute to 10 liters. Allow to stand until the initial rotation of $+5° 18'$ goes up to $+11°$. Bourquelot has obtained a number of similar compounds by this method.

Estimation of Maltase Activity. The colorimetric method by Tauber and Kleiner (43) permits the estimation of monoses in the presence of bioses. The applicability of this method has been amply confirmed (see lactase estimation).

β-d-GALACTOSIDASES: LACTASE, MELIBIASE

Lactase (*β-d-galactosidase*) hydrolyzes lactose to galactose and glucose. Röhmann and Lappe (44, 45, 46) found it to be present in the young mammal's small intestine. According to Porcher (47) it may be prepared from the intestine of the foetus of various animals. Foa (48) stated that the adult mammal's intestinal mucosa contains none or very little lactase. No lactase could be found in the cow's mammary gland by Bradley (49, 49*a*) or by Kleiner and Tauber (30). Recently, however, a lactose-synthesizing action of rabbit's mammary gland tissue has been observed by Michlin and Lewitow (50), and by Grant (51). Cajori (52) examined the intestinal mucosa which he stripped from the duodenum or jejunum of adult dogs. He used the ground fresh tissue as well as the extracts, and also tested the intestinal juice from dogs with Thiry loops and with extracts of the fresh dog liver. Maximum activity was obtained with fresh, finely ground tissues. Liver showed slight activity. The juice from a Thiry loop of the colon did not exhibit lactase activity. These experiments show that lactose is hydrolyzed before it is absorbed. The slight activity of the aqueous extracts and the succus entericus indicates that this enzyme is intimately bound to the mucosal cells. Cajori (53) has also shown that the succus entericus, as a digestive fluid, is of only minor importance, since several enzymes are present only in traces. The major enzymic action results from direct contact with the mucosa or intracellularly.

The digestive tract of *Helix pomatia* has the ability to break down lactose (54). Lactase also occurs in almonds. Hofmann (55) obtained enzyme preparations from *B. coli* and *B. delbruckii* which contained β-*d*-galactosidase, which was free of β-*d*-glucosidase. This is not the case with the almond *β-d*-galactosidase. Lactose yeasts, kafir grains, and certain molds also contain lactase.

Optimum Activity. Dog intestinal lactase (Cajori) has an optimum activity at *p*H 5.4 to 6.0; calf intestinal lactase has an optimum *p*H of 5 (Freudenberg and Hoffmann) (56); and the gut lactase of the cockroach of 5.0 to 6.4 (Wigglesworth) (57). Yeast lactase, however, has an optimum *p*H of 7.0 (Willstätter and Oppenheimer) (58), whereas almond lactase has an optimum of *p*H 4.2.

Reaction Course. Armstrong (59) found that hydrolysis of lactose by the lactase of the kafir-grains follows at first the course of a monomolecular reaction and later decreases. Similar results were obtained by Willstätter and Oppenheimer with yeast lactase and by Cajori with the intestinal lactase of the adult dog (Table XV).

TABLE XV

LACTOSE HYDROLYSIS BY INTESTINAL LACTASE

t	x (lactose hydrolyzed)	K *
hr.	per cent	
1.0	4.0	0.0177
2.0	8.3	0.0188
3.0	12.3	0.0190
5.0	19.5	0.0188
7.0	25.5	0.0183
23.5	62.5	0.0181

* $K = (1/t) \log (100/[100 - x])$.

Effect of Substrate Concentration. The initial velocity of lactose hydrolysis decreases when the concentration of lactose is less than 2 per cent (0.056 *M*). At a concentration of 0.006 *M*, the initial velocity is about one-half the maximum obtained with higher lactose concentrations. Analysis of the results indicates that 0.006 *M* may be regarded as a true Michaelis constant and that 1 molecule of lactose and 1 molecule of enzyme combine during lactase action. Lineweaver and Burk (60) suggest in the simplest case of enzyme substrate, combinations $S + E = SE$ for the evaluation of the dissociation constant K_s, the use of the linear form of the Michaelis-Menten equation

$$1/v = K_sV_{\text{max.}}S + I/V_{\text{max.}}$$

The reciprocals of the velocities (v), plotted against the reciprocal of the lactose concentrations (S), resulted in a straight line. According to the method of Lineweaver and Burk, the slope of the line $K_s/V_{\text{max.}}$ was obtained by straight line extrapolation. In Experiment 1, K_s was calculated as 0.0055, and in Experiment 2 as 0.006. The initial velocities at various lactose concentrations, and initial velocities calculated from the K_s values 0.0055 and 0.0061, are given in Table XVI. Since the observed and

TABLE XVI

INITIAL VELOCITY OF LACTOSE HYDROLYSIS AT DIFFERENT LACTOSE CONCENTRATIONS

	Lactose concentration	Lactose hydrolyzed in 4 hr.	Relative initial velocity observed	Initial velocity calculated $K_s = 0.0055$
	M	mg.	per cent	per cent
Experiment 1..	0.110	24.3	100	
	0.056	24.4	100	
	0.028	22.5	92	84
	0.014	17.5	72	72
	0.009	15.0	62	62
	0.007	14.4	59	56
	0.0035	9.3	38	39
	0.002	6.0	25	27
				$K_s = 0.0061$
Experiment 2..	0.056	30.5	100	
	0.014	23.1	76	70
	0.006	15.0	49	49
	0.0044	11.9	39	42
	0.0017	5.0 *	16	22

* Not used in the calculation of K_s.

calculated velocities harmonize, the extension of the Michaelis-Menten theory to this enzyme is justified.

Estimation of Lactase Activity. In many earlier enzyme studies, the analytical methods used were inadequate. This apparently was also true of lactase studies since it was difficult to determine small amounts of glucose and galactose in the presence of large quantities of lactose. In the above experiments of Cajori (52) and those of other recent investigators (33, 61) the calorimetric method of Tauber and Kleiner (43) for the estimation of monosaccharides in the presence of disaccharides was employed, apparently with great success.

Purification. No special purification methods are available. Cajori found lactose to be adsorbed by aluminum hydroxide or ferric hydroxide from slightly acid solution.

Melibiase. Melibiase splits melibiose into cane sugar and galactose (62). It is found in almonds, bottom yeast, and many plants. The melibiase has an optimum *p*H of 5.5.

EMULSIN

The Specificity of Emulsin. That the bitter almonds contain an enzyme which can split the glucoside amygdalin of the same plant into glucose, benzaldehyde, and HCN has been known for more than 100 years (Robiquet, Liebig, and Wöhler). In 1894 Emil Fischer (1, 1*a*) noticed that one of his synthetic glucosides, β-methyl-*d*-glucoside, was hydrolyzed by emulsin. Since that time all glucosides (natural and synthetic) which can be hydrolyzed by emulsin are called β-*d*-glucosides. The enzyme responsible for this reaction is called β-*d*-glucosidase. It was believed that the β-*d*-glucosidase was not a single enzyme (63, 64). Now, however, it is an established fact that all β-*d*-glucosides may be hydrolyzed by the enzyme present in emulsin, regardless of the nature of the aglucon of the glucoside (65, 66).

Not all β-*d*-glucosides are hydrolyzed equally fast by the *β-d*-glucosidase. Some are split only with difficulty, requiring much more time than others. This "relative specificity" is influenced by the aglucon of the glucoside. Weidenhagen (16) extends this theory by saying that one and the same enzyme (*β-d*-glucosidase of emulsin or other source) hydrolyzes not only all *β-d*-glucosides, but also all oligosaccharides with a *β-d*-glucosidic linkage such as cellobiose and gentiobiose. In the paper of E. Fischer, it was shown that *α*-methyl-*d*-glucoside cannot be split by emulsin. In yeast, however, as has been discussed previously, an enzyme *α-d*-glucosidase, which can hydrolyze *α*-methyl glucoside, is present in abundance. A change in the configuration of the glucoside renders the enzyme *absolute-specific*. *l*-Glucosides are not hydrolyzed by emulsin.

Other glucosides have also been hydrolyzed by emulsin, and this was explained as due to the action of separate enzymes. An *α-l*-arabinosidase was found in emulsin (67, 68), *β*-methyl-*d*-maltoside was hydrolyzed into maltose, and methanol (69, 70) and *β*-methyl-*d*-isorhamnoside were likewise split. These experiments showed that, for the specificity of the enzyme, a change in the constitution of the glucoside molecule is less important than a change in the configuration. Certain *h*-glucosides, *l*-rhamnosides, and *d*-fructosides could not be hydrolyzed by emulsin, nor could the *β-d*-xylosides, which are closely related to the *β-d*-glucosides (1, 1*a*, 71, 72).

From the earlier experiments, many of which have been repeated and extended by Helferich and associates, as well as the new researches (65), it may be said that emulsin of sweet almonds hydrolyzes *β-d*-glucosides and their 6-bromhydrines, *β-d*-isorhamnoside, *β-d*-xyloside, *β-d*-galactoside, and *α-l*-arabinoside. According to Helferich, all these substrates are split by one enzyme, but he believes that this may be disproved. *α-d*-Mannoside, *α-d*-galactoside, and *β-l*-arabinose, according to this investigator, are hydrolyzed by a special enzyme.

The respective substrates are attacked if an exchange of the aglucon takes place. The aglucon may even be substituted for a sugar (primverose) (73); (vicianose) (74). The hydrolysis of phenol-*α*-*d*-galactoside (75) is in accordance with the theory of Weidenhagen (16). That melibiose, a *α*-*d*-galactoside, is hydrolyzed by emulsin is well known (76, 77).

Assuming that all these findings are correct, contrary to earlier belief, sweet almond emulsin (i.e., the main enzyme of it) possesses only a relative specificity towards a number of glucosides with a varying sugar component of the type of *β*-*d*-glucosides.

The Effect of Neutral Salts upon Emulsin. Helferich and Schmitz–Hillebrecht (78) recently made a new and quite remarkable observation concerning the effect of neutral salts upon sweet almond emulsin. These investigators found that neutral salts increase the activity of emulsin, using *β*-glucosides as substrates. The anions are the influencing factors. Cations are without effect. In some cases the increase in activity is as much as 300 per cent (phenol-*β*-*d*-glucoside). Ammonium perchlorate and potassium thiocyanate are the most effective salts. All *β*-*d*-glucosides and all *β*-*d*-xylosides should be hydrolyzed by the same enzyme; the activation by $NaClO_4$, however, differs greatly for the various glucosides of these groups. No definite explanation can be given for this new discovery.

Preparation of Emulsin. Josephson (79) obtained quite active preparations by adsorption with kaolin and dialysis of the ammoniacal elution. Helferich, Winkler, Gootz, Peters, and Günther (80) purified sweet almond emulsin by precipitation with $ZnSO_4$ and tannin and removing the enzyme by extraction, with water and acetone. The resulting extract, which was quite active, was then further purified by precipitation with Ag_2O and treatment with H_2S. This second step yielded a more active preparation. A practical method for the preparation of emulsin has been described by Tauber (81).

POLYASES

Amylases

Amylases are found in saliva, pancreatic juice, blood cells, blood serum, liver, other organs, and many plants. Amylases break down starches and glycogen. Under favorable conditions, the end product of hydrolysis is maltose. The breaking down of starch by amylase, however, is much more complicated than this. The fact that the structure of the starch molecule is unknown complicates the problem, and, as with proteins and other unknown substrates, enzyme action is used as an aid in structure studies.

If a small amount of amylase be added to starch paste, decrease in viscosity, disappearance of the characteristic blue color with iodine, and formation of reducing sugar (maltose) take place. The last may be followed by measuring the increase in reducing power. These changes, however, do not always take place in the same order or at the same rates. Sometimes there is a rapid liquefaction paralleled by a slow maltose formation; at other times, there is a very rapid formation of maltose but slow disappearance of products which give a blue color with iodine. Because of these irregularities, it has been assumed that there must be several amylases.

The properties of malt amylase, because of its importance in the industry, have been known for many years. As early as 1878, Märcker (82) noticed that malt amylase must be a mixture of two components, i.e., a liquefying and a maltose-forming fraction. Somewhat later, others (83, 84) were of the same opinion. Since they could not be completely separated, the existence of two separate enzymes was questioned up to recent times (85–89*a*). Philoche (90) has shown that the pancreatic amylase differs specifically from the maltamylase, since glycogen is only slightly attacked by the latter, but readily hydrolyzed by the former, enzyme. Kuhn has shown that the reaction products of these two enzymes differ.

Kuhn's Amylase Specificity Theory

Kuhn (91) studied the mutarotation of the products of the early stage of starch hydrolysis. He made the important observation that one group of amylases (pancreatic and salivary amylase) yields primarily α-maltose which mutarotates downward. He named this the α-type. Another group (amylases of sprouted and unsprouted grains) he found to yield β-maltose, which mutarotates upward. These amylases he called the β-type. Since it is well known that on hydrolysis of α- and β-glucosides by α- and by β-glucosidases, respectively, α- and β-glucose are liberated, Kuhn believed it evident that the two kinds of amylases split α- and β-linkages in the starch molecule. According to Kuhn, starch is composed of a chain of alternating α- and β-glucosidic linkages. Kuhn also found that β-amylases are much more inhibited by β-maltose than by α-β-maltose, whereas with α-amylases, the degree of inhibition by the two maltoses is reversed.

These findings of Kuhn were corroborated by Weidenhagen and Wolff (92) and extended by Ohlsson (93) and by Reichel (94).

The Two-Starch-Component Theory of Van Klinkenberg

According to Van Klinkenberg (95), starch is a mixture of 36 per cent α-starch and 64 per cent β-starch. α-Starch is converted by α-amylase to maltose, whereas the β-amylase digests the β-starch. Only the α-starch gives a colored compound with iodine. This theory of *two starch components* is of course contrary to the theory of Kuhn, who, as has been stated, believes that starch is a single molecule with a chain of alternating α- and β-glucosidic linkages. Kuhn's theory, however, is supported by the experiments of Hudson and Yanovsky (96), who found that mutarotating maltose at equilibrium consists of 64 per cent β-maltose and 36 per cent α-maltose. Similarly, glucose at equilibrium contains 64 per cent β-glucose and 36 per cent α-glucose.

Lyo- and Desmo-Amylases of Willstätter and Rohdewald

Willstätter and Rohdewald (97) obtained, from various organs of the pig, eight individual amylases, four lyo-(water-soluble) and four desmo-amylases (water-insoluble), which are divided into four subdivisions. Group 1 is inhibited by glycerol and does not need phosphates. Group 2 is inhibited by glycerol and requires phosphates. Group 3 is not inhibited by glycerol and does not require phosphates. Group 4 is not inhibited by glycerol and requires phosphates. These influences are not due to changes in the "colloidal carrier" but rather to changes in the groupings in the enzyme molecule. These organ amylases belong to the α-type of Kuhn.

The Influence of Salts upon the Optimum *p*H of Amylases. Other Activators

Nasse (98) was the first to observe that animal amylases become more active in the presence of neutral salts. Later it was found that pancreatic amylase loses its activity on dialysis and becomes active again on the addition of salts and that malt amylase does not depend on neutral salts (99, 100). According to Haehn and Schweigart (101), potato amylase becomes also reversibly inactive on dialysis just like pancreatic amylase. Sherman, Caldwell, and Cleaveland (102), working under carefully controlled conditions, have recently shown that for the activity of malt amylase neutral salts are not essential. Sherman, Caldwell, and Adams (103) found that the effect of neutral salts on pancreatic amylase does not depend on the purity of the enzyme but is rather a property of the enzyme. Neutral salts are necessary to the activity of pancreatic amylase. Anions, however, are far more influential than the cations.

Chloride ion is the most effective, and the magnitude of activation may be placed in the following order: NaCl, KCl, LiCl, NaBr, $NaNO_3$, $NaClO_3$, NaCNS, NaF. No effect on the activity of pancreatic amylase was shown by Na_2SO_4 and Na_2HPO_4. This is additional evidence that malt amylase and pancreatic amylase are distinct individual enzymes.

Malt amylases have an optimum *p*H at 4.3 to 4.6 in 0.01 *M* acetate buffer at 40° for the saccharogenic activity. Under similar conditions, the amyloclastic action has about the same optimum *p*H (104). Contrary to earlier findings (105, 106), Sherman, Caldwell, and Dale (107) showed that phosphate has no influence upon the activity of pancreatic amylase. Sherman, Caldwell, and Adams (103) studied the influence of salt concentrations on the optimum *p*H and the optimal concentration of each salt at the optimum *p*H. Table XVII shows that the optimum *p*H differs as the NaCl concentration is increased from 0.0005 to 0.01 *M*. Above 0.01 *M*, the optimum *p*H is the same (7.1 to 7.2). With other salts similar results were obtained, but the most favorable concentration depends upon the salt. Sodium sulfate, however, was without influence. Thus the optimum *p*H varied from 6.3 to 7.2. The dependence on these conditions is much more pronounced with pancreatic amylase than with other amylases. In contrast to the optimum of plant amylases, that of animal amylase is closer to the neutral point. The amylase of *Aspergillus oryzae* is most active at *p*H 5.3 to 5.5 in acetate buffer (108).

Myrbäck (109) found that the *p*H-activity curves of pancreatic amylase are identical with those of salivary amylase in the presence of phosphate, chloride, nitrate, and chlorate. Liver amylase is more active in the presence of NaCl (110), and dialyzed salivary amylase is completely inactive on dialyzed starch (111).

Ascorbic Acid. Recently Purr (112) reported that ascorbic acid is a specific activator for the β-type of animal amylase (amylase of human saliva, pig pancreas, and

TABLE XVII

A SUMMARY OF RESULTS WITH DIFFERENT SALTS SHOWING THE INTERRELATIONSHIP BETWEEN CONCENTRATION OF SALT AND HYDROGEN-ION ACTIVITY (EXPRESSED AS *p*H) IN THEIR INFLUENCE UPON THE ACTIVITY OF PANCREATIC AMYLASE *

Concentration of salt, *M*	Most favorable hydrogen-ion activity for pancreatic amylase in the presence of different concentrations of each of the following salts, *p*H						
	NaCl	KCl	NaBr	$NaNO_3$	$NaClO_3$	NaSCN	NaF
0.0005	6.5						
.001	6.7						
.0025	6.9						
.005	7.0	7.0–7.1	......	6.6–6.8	6.5		
.01	7.1	7.1–7.2	7.1	6.9–7.1			
.02	7.1						
.03	7.1	7.1–7.2					
.05	7.1	7.1–7.2	7.1	7.0–7.2	6.9–7.1	6.5	
.10	7.1	......	7.1	......	......	6.7–6.8	6.3–6.7
.15	......	......	......	......	......	6.7–6.8	
.20	......	......	7.1	7.1–7.2	6.9–7.1	6.7–6.8	6.6–6.8
.30	......	......	......	......	......	......	6.6–6.8

* Mixtures of acid and alkaline sodium phosphates corresponding to a total concentration of 0.01 *M* phosphate were present in all cases.

rabbit liver). The β-amylase of plants (barley malt) is inhibited by the ascorbic acid. The α-amylase is not affected by this acid. The oxidized form of ascorbic acid does not affect the β-form but inhibits the α-amylase of plants. β-Amylase of animal tissue occurs in an almost inactive state. In the early stages of the sprouting grain, the same amylase relationship exists. The observations of Virtanen and coworkers (113) that the α-components become inactive at the end of the growth period are verified and explained by the ascorbic acid changes during this period.

Amylokinase. A specific activator, amylokinase, has been obtained from malt extracts by Waldschmidt-Leitz and Purr (114, 114*a*). In unsprouted wheat kernels, amylase is present in an inactive state until sprouting starts, with the formation of amylokinase, which activates the amylase. Weidenhagen (115), however, has shown that amylokinase is not specific since it does not increase the activity of a highly purified malt amylase, but appears to remove inhibitors from the crude enzyme preparation. The purified preparations of malt amylases of Caldwell and Doebbeling were exceedingly active, although no amylokinase was added.

Kinetics of Starch Hydrolysis. According to Willstätter, Waldschmidt-Leitz, and Hesse (116), hydrolysis of starch by pancreatic amylase follows the course of a monomolecular reaction up to a saccharification of about 40 per cent. The attainable limit of digestion is taken as a basis of calculation.

Hanes (117) studied the effect of starch concentration upon the reaction velocity, using the amylase of germinated barley. He observed that the relationship as determined by an initial slope method is in close agreement with that predicted by the Michaelis-Menten theory. The relationship between initial reaction velocity and enzyme concentration is linear over a wide range of enzyme concentration.

Methods for the Estimation of Amylolytic Activity

As with other enzymes, the action of amylases is followed by measurements of the changes which they bring about in suitably prepared substrates. Such measurements include: (*a*) determinations of reducing sugar (maltose) formed (118, 119); (*b*) measurements of the decrease in viscosity of the reaction mixtures (120); (*c*) observations of the changes in color given by the reaction mixtures when treated with iodine (121); and (*d*) determinations of residual amyloses (122).

The choice of method naturally depends upon the circumstances and aim of the work. It has become increasingly evident, however, that studies of the nature of amylases and their action should include quantitative comparisons of different kinds of activity measurements as each type of procedure appears to yield information concerning amylases which is not afforded by the others. It has already been pointed out, however, that such comparisons have led to the conclusion that there are at least two kinds of amylases in extracts of barley malt.

Purification of Pancreatic Amylase and Its Chemical Nature

In 1911 Sherman and Schlesinger (123) reported on the preparation of a highly active pancreatic amylase. It had a constant activity in many independent purification experiments. In the dry state this amylase was an amorphous white powder having the composition and showing the color tests of a typical protein. This purified pancreatic amylase had the highest activity ever recorded up to that time. In thirty minutes at 40° it hydrolyzed 20,000 times its weight of starch and formed 10,000 times its weight of maltose. The enzyme was still quite active at a dilution of 1 to 100,000,000, whereas the most delicate tests for protein are not valid at a dilution greater than 1 to 100,000 showing that failure of protein reactions in active enzyme solutions does not prove, as many writers assume, that the enzyme is of other than protein nature (124). Willstätter, Waldschmidt-Leitz, and Hesse (116) in 1922 took up the study of pancreatic amylase. They questioned the conclusions of Sherman and his associates, stating that their preparations were free of protein. The pancreatic extract of Willstätter and coworkers, however, was dialyzed against running water for a considerable time, thus exposing it to favorable conditions of tryptic digestion and testing their inactive amylase for protein. They assumed that the substance of the enzyme (although inactivated) might be expected to remain in the dialyzing bag.

Recently Sherman, Caldwell, and Adams (125) developed a new method for the purification of pancreatic amylase. This method involves adsorption by alumina gel and subsequent precipitation by alcohol and ether. At *p*H 5.2 to 6.0 the amylase is readily adsorbed by alumina gel, and at *p*H 7.3 it may be extracted from the alumina gel. This is in accordance with the view that pancreatic amylase is amphoteric. The yields of amylase are larger and the enzyme is slightly more active than those obtained by the Sherman-Schlesinger method. The enzyme is a typical protein.

Crystalline Pancreatic Amylase. Pancreatic amylase has been obtained by Caldwell, Booher, and Sherman (126) in crystalline form from buffered aqueous alcohol solutions of highly purified preparations. The crystals are protein and are very active.

More recently, Waldschmidt-Leitz and Reichel (127) repeated the work of Willstätter and coworkers and stated that they too obtained pancreatic amylase free of protein. Judging, however, from the dry weight and activity of their preparation, it is obvious that the enzyme solution was too dilute to give positive protein color tests.

Separation of α- and β-Malt Amylase and Their Purification

Sherman, Caldwell, and Doebbeling (128) obtained β-amylase practically free of α-amylase, whose amyloclastic activity was negligible. Their method was repeatedly to fractionate extracts of barley malt with ammonium sulfate followed by dialysis and fractional precipitation with alcohol. It had much higher saccharogenic activity than any previously reported malt amylase. Lüers and Sellner (129) have also obtained highly active preparations of malt amylase. Caldwell and Doebbeling (129*a*) have examined the various fractions for both types of activities and found that in the early stages of ammonium sulfate fractionation the precipitates which are formed by low alcohol concentrations are high in amyloclastic activity. These fractions had been discarded previously because they had slight activity when judged by their maltose-forming action. It was found that these fractions have about 30 times more amyloclastic activity than the original malt extracts, and that the separation does not involve a loss in active material. This is the first time that highly active preparations

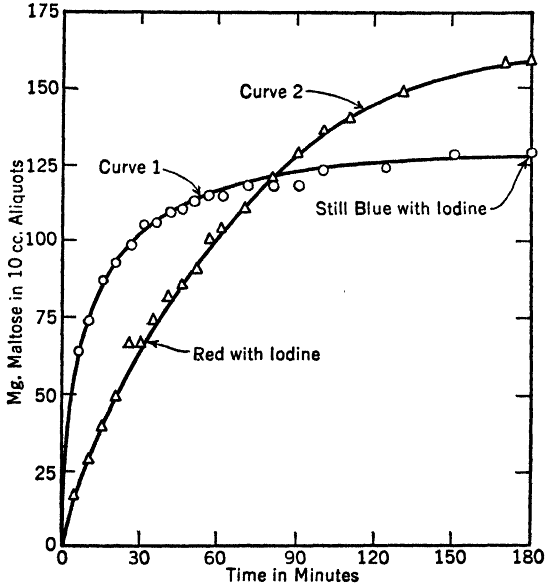


Fig. 21.—Course of hydrolysis of starch by two types of malt amylase preparations

of the two kinds of malt amylases have been simultaneously obtained from a single source. Both are of protein nature. Figure 21 shows the course of the hydrolysis of starch by the two types of amylase preparations. The reducing values of the digest mixture, expressed as maltose, are plotted against time. The *p*H was 4.5 (2 per cent starch and 0.01 *M* acetate at 40°). This has been found by Caldwell and Doebbeling to be the optimum *p*H for both amylases. It can be seen from Fig. 21 that the two types of amylases hydrolyze starch differently. Curve 1 of Fig. 21 represents the case in which maltose is formed rapidly at first (β-amylase), soon reaches a maximum, and then increases only slowly. Curve 2 represents the other case. Here maltose appears only slowly at first, but its formation is longer maintained (α-amylase).

The rapid maltose production in the early part of hydrolysis as represented by Curve 1 is accompanied by a slow disappearance of products which give a blue color with iodine. Thus, if the iodine test alone were used, the observer would reach the erroneous impression that the preparation is low in amylolytic activity. On the other hand, the slower maltose formation, as affected by the second type of amylase and represented by Curve 2, is accompanied by the rapid disappearance of products which give a blue color reaction with iodine.

Curve 1 represents the following results: Blue color with iodine at 30 minutes and 160 mg. of maltose per 10 cc.; nearing the red end point with iodine at 1300 minutes and 154 mg. of maltose per 10 cc.; no color with iodine at 2700 minutes and 187 mg. of maltose per 10 cc., which is 89 per cent of the theoretical yield of maltose. Curve 2 represents the following experiment: Clear red color with iodine in 30 minutes and 65 mg. of maltose per 10 cc.; no color with iodine in 45 minutes and 85 mg. of maltose per 10 cc.; 209 mg. of maltose per 10 cc. in 1300 minutes. This is the theoretical yield on maltose.

LICHENASE, CELLULASE, INULASE

This group of polyases hydrolyze reserve carbohydrates of the plant framework.

Lichenase. Lichenase hydrolyzes lichenin to cellobiose. It is a reserve carbohydrate closely related to cellulose. For a discussion of the higher carbohydrates, see Haworth (130) and Staudinger (131). Lichenase has been obtained from malt extracts by Pringsheim and associates (132, 133, 134) and from the intestines of snails, as well as from corn, beans, hyacinths, and other plants by Karrer, Toos, and Staub (135).

Cellulase. Karrer, Schubert, and Weholi (136) conducted interesting experiments with snail cellulase, using artificial silk, cellulose, and filter paper as a substrate. The last they found most suitable. Pringsheim and Bauer (137) studied the effect of malt cellulase on chemically treated cellulose. Von Euler (138) noticed cellulase activity by the mushroom *Merculius lacrimans*. Schmitz (139) found cellulase in a great number of molds. The fact that cellulose is broken down by the intestinal tract of higher animals (e.g., straw by cows) is due to certain intestinal bacteria and not to enzymes.

Grassmann, Stadler, and Bender (31) recently studied crude enzyme preparations from fungi. They hydrolyzed cellulose, lichenin, and xylan readily and also to a certain extent hydrated pectin, mannan, and inulin. After dialysis, they attacked only cellulose, lichenin, and xylan. Treatment with charcoal removed the xylanase. The filtrate readily split cellulose and lichenin with an optimum *p*H of 4.5 for each substrate. These authors believe that mannanase, xylanase, and inulase are specific enzymes. Cellulase and lichenase are perhaps one enzyme.

Inulase. Inulase splits inulin into *d*-fructose. Inulase may be best obtained from molds such as *Aspergillus niger* and *Penicillium glaucum* (140). According to Lindner (141) it is also present in baker's yeast. Avery and Cullen (142) discovered inulase in pneumococci.

These polyases are usually found together and are difficult to separate. At the present time, no extensive researches are available concerning this group of enzymes.

REFERENCES

1. Fischer, E.: Bedeutung der Stereochemie für die Physiologie. *Z. physiol. Chem.*, **26**, 60 (1898–99).

1*a*. Fischer, E.: Einfluss der Configuration auf die Wirkung der Enzyme. *Ber.*, **27**, 2985 (1894).

2. Kjeldahl, M. J.: Recherches sur les hydrates de carbone de l'orge et du malt, specialement au point de vue de la présence du sucre de canne. *Meddel. Carlsberg Lab.*, **1**, 189 (1881).
3. Michaelis, L.: Die Adsorptionsaffinitäten des Hefe-Invertins. *Biochem. Z.*, **7**, 488 (1907–08).
4. O'Sullivan, C., and Tompson, F. W.: Invertase: A contribution to the history of an enzyme or unorganised ferment. *J. Chem. Soc.*, **57**, 834 (1890).
5. Hudson, C. S.: The inversion of cane sugar by invertase. II. *J. Am. Chem. Soc.*, **30**, 1564 (1908).
6. Euler, H. von, Lindberg, E., and Melander, K.: Zur Kenntnis der Invertase. Vorläufige Mitteilung. *Z. physiol. Chem.*, **69**, 152 (1910).
7. Euler, H. von, and Kullberg, S.: Versuche zur Reindarstellung der Invertase. *Z. physiol. Chem.*, **73**, 335 (1911).
8. Willstätter, R.: Discussion on enzymes. *Proc. Roy. Soc. London*, *III B*, **282** (1932).
9. Willstätter, R., and Schneider, K.: Zur Kenntnis des Invertins (Achte Abhandlung). *Z. physiol. Chem.*, **142**, 257 (1925).
10. Willstätter, R., and Racke, F.: Invertase. *Ann.*, **425**, 1 (1921).
10*a*. Willstätter, R., and Racke, F.: Invertase. II. *Ann.*, **427**, 111 (1922).
11. Lutz, J. G., and Nelson, J. M.: Preparation of highly active yeast invertase. *J. Biol. Chem.*, **107**, 169 (1934).
12. Willstätter, R., and Wassermann, W.: Zur Kenntnis des Invertins (Vierte Abhandlung). *Z. physiol. Chem.*, **123**, 181 (1922).
13. Willstätter, R., and Schneider, K.: Zur Kenntnis des Invertins (Fünfte Abhandlung). *Z. physiol. Chem.*, **133**, 193 (1924).
13*a*. Nelson, J. M.: Personal communication to the author.
14. Michaelis, L., and Davidsohn, H.: Die Wirkung der Wasserstoffionen auf das Invertin. *Biochem. Z.*, **35**, 386 (1911).
15. Nelson, J. M.: Enzymes from the standpoint of the chemistry of invertase. *Chem. Rev.*, **12**, 1 (1933).
16. Weidenhagen, R.: Spezifität und Wirkungsmechanismus der Carbohydrasen. *Ergebnisse Enzymforschung*, **1**, 168 (1932).
17. Willstätter, R., and Kuhn, R.: Über Masseinheiten der Enzyme. *Ber.*, **56**, 509 (1923).
18. Euler, H. von, and Svanberg, O.: Saccharasegehalt und Saccharasebildung in der Hefe. *Z. physiol. Chem.*, **106**, 201 (1919).
19. Euler, H. von, and Josephson, K.: Bezeichnung der Aktivität und Affinität von Enzymen. *Ber.*, **56**, 1749 (1923).
20. Sumner, J. B., and Howell, S. F.: A method for determination of saccharase activity. *J. Biol. Chem.*, **108**, 51 (1935).

21. Sumner, J. B.: A more specific reagent for the determination of sugar in urine. *J. Biol. Chem.*, **65**, 393 (1925).
22. Nelson, J. M., and Vosburgh, W. C.: Kinetics of invertase action. *J. Am. Chem. Soc.*, **39**, 790 (1917).
23. Fischer, E., and Niebel, W.: Behavior of polysaccharides with certain animal secretions and organs. *Sitzb. preuss. Akad. Wiss. physik. math. Klasse*, **5**, 73 (1896).
24. Weidenhagen, R.: Taka-invertase. *Z. Ver. deut. Zucker-Ind.*, **78**, 25, 242, 406 (1928).

24*a*. Weidenhagen, R.: Specific nature of invertase. *Z. Ver. deut. Zucker-Ind.*, **78**, 406 (1928).

25. Karstroem, H.: Zur Spezifität der α-Glucosidasen. *Biochem. Z.*, **231**, 399 (1931).
26. Myrbäck, K.: α-Glucosidase und Disaccharidspaltung. *Z. physiol. Chem.*, **205**, 248 (1932).
27. Tauber, H., and Kleiner, I. S.: The digestion and inactivation of maltase by trypsin and the specificity of maltases. *J. Gen. Physiol.*, **16**, 767 (1932–33).
28. Pringsheim, H., Borhardt, H., and Loew, F.: Über die Spezifität der Saccharasen. *Z. physiol. Chem.*, **202**, 23 (1931).
29. Iwanoff, N. N., Dodonowa, E. W., and Tschastuchin, W. J.: Die Fermente der Hutpilze. *Fermentforschung*, **11**, 433 (1929–30).
30. Kleiner, I. S., and Tauber, H.: Enzymes of the mammary gland. The presence of glucomaltase in the mammary gland. *J. Biol. Chem.*, **99**, 241 (1932–33).
31. Grassmann, W., Stadler, R., and Bender, R.: Zur Spezifität Cellulose- und Hemicellulose spaltender Enzyme (Erste Mitteilung über enzymatische Spaltung von Polysacchariden). *Ann.*, **502**, 20 (1933).
32. Grassmann, W., Zechmeister, L., Toth, G., and Stadler, R.: Über den enzymatischen Abbau der Cellulose und ihrer Spaltprodukte (Zweite Mitteilung über enzymatische Spaltung von Polysacchariden). *Ann.*, **503**, 167 (1933).
33. Hotchkiss, M.: Evidence on the specificity of hexosidases. *J. Bac.*, **29**, 391 (1935).
34. Tauber, H., and Kleiner, I. S.: Some enzymes of *Solanum indicum*. *J. Biol. Chem.*, **105**, 679 (1934).
35. Kuhn, R., and Münch, H.: Über Gluco- und Fructosaccharase. II. *Z. physiol. Chem.*, **163**, 1 (1927).
36. Leibowitz, J., and Mechlinski, P.: Über Takamaltase und Takasaccharase (Zweiter Beitrag zur Spezifität der Disaccharasen). *Z. physiol. Chem.*, **154**, 64 (1926).
37. Michaelis, L., and Rona, P.: Die Wirkungsbedingungen der Maltase aus Bierhefe. I. *Biochem. Z.*, **57**, 70 (1913).

38. Willstätter, R., and Bamann, E.: Trennung von Maltase und Saccharase (Siebente Mitteilung über Maltase). *Z. physiol. Chem.*, **151**, 273 (1926).

39. Willstätter, R., Kraut, H., and Erbacher, O.: Über Isomere Hydrogele der Tonerde (Siebente Mitteilung über Hydrate und Hydrogele). *Ber.*, **58**, 2448, 2458 (1925).

40. Willstätter, R., Oppenheimer, G., and Steibelt, W.: Über Maltaselösungen aus Hefe. *Z. physiol. Chem.*, **110**, 232 (1920).

40*a*. Willstätter, R., and Steibelt, W.: Über die Verschiedenheit von Maltase und α-Glukosidase (Dritte Mitteilung über Maltase). *Z. physiol. Chem*, **115**, 199 (1921).

41. Aubry, A.: Thèse, Paris, p. 19, 1914.

42. Bourquelot, E.: Biochemical synthesis of α-glucosides of monovalent alcohols. II. α-glucosides. *Ann. chim.*, III, **287** (1915).

43. Tauber, H., and Kleiner, I. S.: A method for the determination of monosaccharides in the presence of disaccharides and its application to blood analysis. *J. Biol. Chem.*, **99**, 249 (1932–33).

44. Röhmann, F., and Lappe, J.: Über die Lactase des Dünndarms. *Ber.*, **28**, 2506 (1895).

45. Portier, P.: Recherches sur la lactase. *Compt. rend. soc. biol.*, **50**, 387 (1898).

46. Bierry, H., and Salazar, G.: Recherches sur la lactase animale. *Compt. rend. soc. biol.*, **57**, 181 (1904).

47. Porcher, C., and Tapernoux, A.: Sur l'apparition de la lactase, dans l'intestin pendant la vie foêtale. *Compt. rend. soc. biol.*, **83**, 420 (1920).

48. Foa, C.: Richerche sulla lattasi intestinale. Contributo allo studio dell'adattamento dei fermenti nell'organismo vivente. *Arch. Fisiologia*, **8**, 121 (1910).

49. Bradley, H. C.: Problem of enzyme synthesis. I. Lipase and fat of animal tissues. *J. Biol. Chem.*, **13**, 407 (1912–13).

49*a*. Bradley, H. C.: IV. Lactase of the mammary gland. *J. Biol. Chem.*, **13**, 431 (1912–13).

50. Michlin, D., and Lewitow, M.: Über die Synthese von Lactose in der Milchdrüse. *Biochem. Z.*, **271**, 448 (1934).

51. Grant, G. A.: The metabolism of galactose. II. The synthesis of lactose by slices of active mammary gland *in vitro*. *Biochem. J.*, **29**, 1905 (1935).

52. Cajori, F. A.: The lactose activity of the intestinal mucosa of the dog and some characteristics of intestinal lactase. *J. Biol. Chem.*, **109**, 159 (1935).

53. Cajori, F. A.: The enzyme activity of dog's intestinal juice and its relation to intestinal digestion. *Am. J. Physiol.*, **104**, 659 (1933).

54. Bierry, H., and Giaja, J.: Sur la digestion des glucosides et du lactose. *Compt. rend. soc. biol.*, **66**, 1038 (1906).
55. Hofmann, E.: Über das Vorkommen von Glucosidasen bzw. Galaktosidasen und Disaccharide spaltenden Enzymen in Bakterien. *Biochem. Z.*, **272**, 133 (1934).
56. Freudenberg, E., and Hoffmann, P.: Lactasestudien. *Klin. Wochsch.*, **1**, 2333 (1922).
57. Wigglesworth, V. B.: Digestion in the cockroach. II. The digestion of carbohydrates. *Biochem. J.*, **21**, 797 (1927).
58. Willstätter, R., and Oppenheimer, G.: Über Lactasegehalt und Gärvermögen von Milchzuckerhefen. *Z. physiol. Chem.*, **118**, 168 (1922).
59. Armstrong, E. F.: Studies on enzyme action. II. The rate of change, conditioned by sucroclastic enzymes, and its bearing on the law of mass action. *Proc. Roy. Soc. London*, **73**, 500 (1904).
60. Lineweaver, H., and Burk, D.: The determination of enzyme dissociation constants. *J. Am. Chem. Soc.*, **56**, 658 (1934).
61. Willstätter, R., and Rohdewald, M.: Über die Amylasen der Leber und anderer Organe. *Z. physiol. Chem.*, **229**, 255 (1934).
62. Neuberg, C.: Zur Kenntnis der Raffinose. Abbau der Raffinose zu Rohrzucker und α-Galaktose. *Biochem. Z.*, **3**, 519 (1907).
63. Willstätter, R., and Oppenheimer, G.: Zur Kenntnis des Emulsins (Zweite Abhandlung). *Z. physiol. Chem.*, **121**, 183 (1922).
64. Willstätter, R., Kuhn, R., and Sobotka, H.: Über die einheitliche Natur der β-Glucosidase des Emulsins. *Z. physiol. Chem.*, **129**, 33 (1923).
65. Helferich, B.: Die Spezifität des Emulsins. *Ergebnisse Enzymforschung*, **2**, 74 (1933).
66. Fischer, E.: Über die Verbindungen der Zucker mit den Alkoholen und Ketonen. *Ber.*, **28**, 1145 (1895).
67. Hudson, C. S.: The significance of certain numerical relations in the sugar group. *J. Am. Chem. Soc.*, **31**, 66 (1909).
68. Bridel, M., and Beguin, C.: Chimie biologique. Synthèse biochimique, a l'aide de l'émulsine des amandes, de l'éthyl-*l*-arabinoside *α*. *Compt. rend. acad. sci.*, **182**, 812 (1926).
69. Fischer, E., and Armstrong, E. F.: Über die isomeren Acetohalogen-Derivate des Traubenzuckers und die Synthese der Glucoside. *Ber.*, **34**, 2885 (1901).
70. Helferich, B., and Becker, J.: Synthese eines Disaccharidglucosids. *Ann.*, **440**, 17 (1924).
71. Fischer, E.: Über den Einfluss der Configuration auf die Wirkung der Enzyme. III. *Ber.*, **28**, 1429 (1895).
72. Fischer, E.: Über die Struktur der beiden Methylglucoside und über ein drittes Methyl-glucosid. *Ber.*, **47**, 1980 (1914).

73. Bridel, M.: Physiologie végétale. Sur la présence, dans l'émulsine des amandes, der deux nouveaux ferments, la primevérosidase et la primevérase. *Compt. rend. acad. sci.*, **181**, 523 (1925).

74 Bertrand, G., and Weisweiller, G.: Chimie organique. Recherches sur la constitution du vicianose: hydrolyze diastasique. *Compt. rend. acad. sci.*, **151**, 325 (1910).

75. Helferich, B., Winkler, S., Gootz, R., Peters, O., and Günther, E.: Über Emulsin. VII. *Z. physiol. Chem.*, **208**, 91 (1932).

76. Fischer, E., and Armstrong, E. F.: Synthese einiger neuer Disaccharide. *Ber.*, **35**, 3144 (1902).

77. Helferich, B., and Bredereck, H.: Zuckersynthesen. Synthesen der Melibiose. *Ann.*, **465**, 170 (1928).

78. Helferich, B., and Schmitz-Hillebrecht, E.: Über Emulsin. XX. Der Einfluss von Neutralsalzen auf die Wirksamheit von Mandelemulsin. *Z. physiol. Chem.*, **234**, 54 (1935).

79. Josephson, K.: Die Enzyme des Emulsins. II. *Ber.*, **59**, 821 (1926).

80. Helferich, B., Winkler, S., Gootz, R., Peters, O., and Günther, E.: Über Emulsin. VII. *Z. physiol. Chem.*, **208**, 91 (1932).

81. Tauber, H.: The chemical nature of emulsin, rennin, and pepsin. *J. Biol. Chem.*, **99**, 257 (1932–33).

82. Märcker, —.: *Chem. Zentr.*, 559 (1878).

83. Dubourfsaut, —.: Mémoire sur la saccharification des fécules. 2 Ed., 140 (1882).

84. Bourquelot, E.: Sur les caractères de l'affaiblissement eprouvé par la diastase sous l'action de la chaleur. *Compt. rend. acad. sci.*, **104**, 576 (1887).

85. Bourquelot, E.: Sur les caractères de l'affaiblissement eprouvé par la diastase (amylase) sous l'action de la chaleur. *Ann. Inst. Pasteur*, **1**, 337 (1887).

86. Hizume, K.: Zur Kenntnis der Diastasen. Zugleich ein Beitrag zur Frage der Zweienzymtheorie. *Biochem. Z.*, **146**, 52 (1924).

87. Nishimura, S.: Über einen Aktivator der Malzamylase. *Biochem. Z.*, **200**, 81 (1928).

88. Sabolitschka, T.: Über die Malzamylase. V. Bestimmung der dextrinierenden und verzuckernden Wirkung der Amylase und Vergleich beider Wirkungen von Sabalitschka, T., und Weidlich, R. *Biochem. Z.*, **207**, 476 (1929).

89. *Idem:* Über die Malzamylase. VIII. Einheit des Dextrinierungs und Verzuckerungsenzyms von Sabalitschka, T., und Weidlich, R. *Ibid.*, **215**, 267 (1929).

89*a*. *Idem:* Über die Malzamylase. VII. Adsorption der Amylase aus

Malzauszugen an Kaolin und Elution von Sabalitschka, T., und Weidlich, R. *Ibid.*, **211**, 229 (1929).

90. Philoche, C.: Recherches physico-chimie sur l'amylase et la maltase. *J. chim. phys.*, **6**, 355, 394 (1908).
91. Kuhn, R.: Der Wirkungsmechanismus der Amylasen; ein Beitrag zum Konfigurationsproblem der Stärke. *Ann.*, **443**, 1 (1925).
92. Weidenhagen, R., and Wolff, A.: Zur Kenntniss der Stärke. III. *Z. Ver. Zucker-Ind.*, **80**, 944 (1930).
93. Ohlsson, E.: Über die beiden Komponenten der Malzdiastase, besonders mit Rücksicht auf die Mutarotation der bei der Hydrolyze der Stärke gebildeten Produkte. Dreizehnte Untersuchung der Mutarotation der Hydrolyseprodukte. *Z. physiol. Chem.*, **189**, 57 (1930).
94. Reichel, M.: Dissertation, Deutsche techn. Hochschule. Prague, 1932.
95. Klinkenberg, van, G. A.: Das Spezifitätsproblem der Amylasen. *Ergebnisse Enzymforschung*, **3**, 73 (1934).
96. Hudson, C. S., and Yanovsky, E.: Indirect measurements of the rotary powers of some alpha and beta forms of the sugars by means of solubility experiments. *J. Am. Chem. Soc.*, **39**, 1031 (1917).
97. Willstätter, R., and Rohdewald, M.: Über die Amylasen der Leukocyten. II. Neunte Abhandlung über Enzyme der Leukocyten. *Z. physiol. Chem.*, **221**, 13 (1933).
98. Nasse, O.: Untersuchungen über die ungeformten Fermente. *Pflügers Arch.*, **11**, 138 (1875).
99. Bierry, H., and Giaja, J.: Chimie physiologique. Sur l'amylase et la maltase du suc pancréatique. *Compt. rend.*, **143**, 300 (1906).
100. Eadie, G. S.: On liver amylase. *Biochem. J.*, **21**, 314 (1927).
101. Haehn, H., and Schweigart, H.: Zur Kenntnis der Kartoffelamylase (Zerlegung in eine organische Komponente und Neutralsalze). *Biochem. Z.*, **143**, 516 (1923).
102. Sherman, H. C., Caldwell, M. L., and Cleaveland, M.: The influence of certain neutral salts upon the activity of malt amylase. *J. Am. Chem. Soc.*, **52**, 2436 (1930).
103. Sherman, H. C., Caldwell, M. L., and Adams, M.: A quantitative comparison of the influence of neutral salts on the activity of pancreatic amylase. *J. Am. Chem. Soc.*, **50**, 2538 (1928).
104. Caldwell, M. L., and Doebbeling, S. E.: A study of the concentration and properties of two amylases of barley malt. *J. Biol. Chem.*, **110**, 739 (1935).
105. Hahn, A., and Michalik, R.: Über den Einfluss neutraler Alkalisalze auf diastatische Fermente (Dritte Mitteilung). *Z. Biol.*, **73**, 10 (1921).

106. Hahn, A., and Meyer, H.: Über den Einfluss neutraler Alkalisalze auf diastatische Fermente (Fünfte Mitteilung). *Z. Biol.*, **76**, 227 (1922).

107. Sherman, H. C., Caldwell, M. L., and Dale, J. E.: A quantitative study of the influence of sodium acetate, sodium borate, sodium citrate and sodium phosphate upon the activity of pancreatic amylase. *J. Am. Chem. Soc.*, **49**, 2596 (1927).

108. Caldwell, M. L., and Tyler, M. G.: A quantitative study of the influence of acetate and of phosphate upon the activity of the amylase of *Aspergillus oryzae*. *J. Am. Chem. Soc.*, **53**, 2316 (1931).

109. Myrbäck, K.: Über Verbindungen einiger Enzyme mit inaktivierenden Stoffen. *Z. physiol. Chem.*, **159**, 1 (1926).

110. Eadie, G. S.: On liver amylase. *Biochem. J.*, **21**, 314 (1927).

111. Michaelis, L., and Pechstein, H.: Die Wirkungsbedingungen der Speicheldiastase. *Biochem. Z.*, **59**, 77 (1914).

112. Purr, A.: The influence of vitamin C (ascorbic acid) on plant and animal amylase. *Biochem. J.*, **28**, 1141 (1934).

113. Virtanen, A. I., Hausen, S., and Saastamoinen, S.: Untersuchungen über die Vitaminbildung in Pflanzen. I. *Biochem. Z.*, **267**, 179 (1933).

114. Waldschmidt-Leitz, E., and Purr, A.: Über Amylokinase, einen natürlichen Aktivator des Stärkeabbaues in keimender Gerste (Zweite Mitteilung über enzymatische Amylolyse in der von M. Samec und E. Waldschmidt-Leitz begonnenen Untersuchungsreihe). *Z. physiol. Chem.*, **203**, 117 (1931).

114*a*. *Idem:* Zur Kenntnis der Amylokinase (Dritte Mitteilung über enzymatische Amylolyse in der von M. Samec und E. Waldschmidt-Leitz begonnenen Untersuchungsreihe). *Ibid.*, **213**, 63 (1932).

115. Weidenhagen, R.: Über die Reinigung pflanzlicher Amylasen. *Z. Ver. Zucker-Ind.*, **83**, 505 (1933).

116. Willstätter, R., Waldschmidt-Leitz, E., and Hesse, A. R. F.: Über Pankreas-Amylase (Dritte Abhandlung über Pankreasenzyme). *Z. physiol. Chem.*, **126**, 143 (1923).

117. Hanes, C. S.: Studies on plant amylases. The effect of starch concentration upon the velocity of hydrolysis by the amylase of germinated barley. *Biochem. J.*, **26**, 1406 (1932).

118. Sherman, H. C., Kendall, E. C., and Clark, E. D.: Studies on Amylases. I. An examination of methods for the determination of diastatic power. *J. Am. Chem. Soc.*, **32**, 1073 (1910).

119. Sherman, H. C., and Baker, J. G.: Experiments upon starch as substrate for enzyme action. *J. Am. Chem. Soc.*, **38**, 1885 (1916).

120. Jozsa, S., and Gore, H. C.: Determination of liquefying power of malt diastase. *Ind. Eng. Chem., Anal. Ed.*, **2**, 26 (1930).

121. Wohlgemuth, J.: Über eine neue Methode zur quantitaven Bestimmung des diastatischen Ferments. *Biochem. Z.*, **9**, 1 (1908).

122. Caldwell, M. L., and Hildebrand, F. C.: A method for the direct and quantitative study of amolyclastic activity of amylases. *J. Biol. Chem.*, **111**, 411 (1935).

123. Sherman, H. C., and Schlesinger, M. D.: Studies on amylases. III. Experiments upon the preparation and properties of pancreatic amylase. *J. Am. Chem. Soc.*, **33**, 1195 (1911). *Idem:* Studies on amylases. IV. A further investigation of the properties of pancreatic amylase. *Ibid.*, **34**, 1104 (1912).

124. Sherman, H. C., Caldwell, M. L., and Adams, M.: Further experiments upon the purification of pancreatic amylase. *Proc. Soc. Exptl. Biol. Med.*, **23**, 413 (1926).

125. Sherman, H. C., Caldwell, M. L., and Adams, M.: Enzyme purification. Further experiments with pancreatic amylase. *J. Biol. Chem.*, **88**, 295 (1930).

126. Caldwell, M. L., Booher, L. E., and Sherman, H. C.: Crystalline amylase. *Science*, **74**, 37 (1931).

127. Waldschmidt-Leitz, E., and Reichel, M.: Zur Frage nach der chemischen Natur der Pankreasamylase (Achtzehnte Abhandlung über Pankreasenzyme in der von R. Willstätter und Mitarbeitern begonnenen Untersuchungsreihe). *Z. physiol. Chem.*, **204**, 197 (1932).

128. Sherman, H. C., Caldwell, M. L., and Doebbeling, S. E.: Further studies upon the purification and properties of malt amylase. *J. Biol. Chem.*, **104**, 501 (1934).

129. Lüers, H., and Sellner, E.: Purification of malt amylase. *Wochenschr. Brauerei*, **42**, 97, 103, 110 (1925).

129*a*. Caldwell, M. L., and Doebbeling, S. E.: A study of the concentration and properties of two amylases of barley malt. *J. Biol. Chem.*, **110**, 739 (1935).

130. Haworth, W. N.: Die Konstitution der Kohlenhydrate. Dresden, 1932.

131. Staudinger, H.: Die hochmolekularen organischen Verbindungen. Berlin, 1932.

132. Pringsheim, H., and Seifert, K.: Über die fermentative Spaltung des Lichenins (Dritte Mitteilung über Hemicellulosen). *Z. physiol. Chem.*, **128**, 284 (1923).

133. Pringsheim, H., and Leibowitz, J.: Über Cellobiase und Lichenase (Vierte Mitteilung über Hemicellulosen). *Z. physiol. Chem.*, **131**, 262 (1923).

134. Pringsheim, H., and Kusenack, W.: Über Lichenin und die Lichenase (Fünfte Mitteilung über Hemicellulosen). *Z. physiol. Chem.*, **137**, 265 (1924).

135. Karrer, P., Toos, B., and Staub, M.: Polysaccharide. XXI. Zur Kenntnis des Lichenins. II. *Helv. Chim. Acta*, **6**, 800 (1923). *Idem:* Polysaccharide. XXII. Zur Kenntnis des Lichenase, und Reserve cellulose (Lichenin). *Ibid.*, **7**, 144 (1924). *Idem:* Polysaccharide. XXVI. Zur Spaltung des Lichenins in Glucose. *Ibid.*, **7**, 518 (1924).
136. Karrer, P., Schubert, P., and Weholi, W.: Polysaccharide. XXXIII. Über enzymatischen Abbau von Kunstseide und nativer Cellulose. *Helv. Chim. Acta*, **8**, 797 (1925). *Idem:* Polysaccharide. XXXV. Weitere Beiträge zum enzymatischen Abbau der Kunstseide und nativer Cellulose. *Ibid.*, **9**, 893 (1926).
137. Pringsheim, H., and Bauer, K.: Über die Spaltung von Lichenin und Cellulose durch die Fermente des Gerstenmalzes. *Z. physiol. Chem.*, **173**, 188 (1928).
138. Euler, H. von: Zur Kenntnis der Cellulose. *Z. angew. Chem.*, **25**, 250 (1912).
139. Schmitz, H.: Studies in wood decay. II. Enzyme action in *Polyporus volvatus* Peck and *Fomes igniarius* (L.) Gillet. *J. Gen. Physiol.*, **3**, 795 (1920–21).
140. Bourquelot, E.: Chimie organique. Inulase et fermentation alcoolique indirecte de l'inuline. *Compt. rend. acad. sci.*, **116**, 1143 (1893).
141. Lindner, P.: Gärversuche mit verschiedenen Hefe und Zuckerarten. *Woch. Brau.* (1900).
142. Avery, O. T., and Cullen, G. E.: Studies on the enzymes of pneumococcus. III. Carbohydrate-splitting enzymes: invertase, amylase, and inulase. *J. Exptl. Med.*, **32**, 583 (1920).

CHAPTER VI

CATALASE

Catalase decomposes hydrogen peroxide into water and inert molecular oxygen. It also attacks monoethyl hydrogen peroxide but much more slowly. One of the split products is acetaldehyde. Peroxides and their organic derivatives are split by peroxidase. Catalase is found in most plant and animal tissues. Physiologically, it is very important, since it decomposes hydrogen peroxide, a toxic substance, and at the same time it furnishes oxygen for dehydrogenation. It seems that catalase is an indispensable constituent of aerobic cells.

Preparation

Although catalase can be prepared from practically any cell, horse liver is the best source. Von Euler and Josephson (1) obtained very active preparations by fractional precipitation of H_2O extracts with alcohol, adsorption, and dialysis. The method of Zeile and Hellström (2) is quite simple and yields hemoglobin-free preparations of high activity.

Some of the liver tissue which has been ground up is extracted with several volumes of H_2O. To 100 cc. of the extract, 50 cc. alcohol are added, centrifuged, one-third of its volume of alcohol added, and shaken with 50 cc. of chloroform. The chloroform precipitates the hemoglobin. The enzyme is adsorbed now from the extract on 1 gram of tricalcium phosphate (3 per cent suspension), and eluted from the adsorbate with several portions of a 1 per cent solution of Na_2HPO_4, using a total of 80 cc. The eluate is dialyzed at a low temperature. The yield is 70 per cent of the original extract. Activity Cat. f. = 30,000 (3).

Zeile and Hellström described methods for the preparation of catalase from germinated squash seeds, and Zeile (2) described its preparation from mold (*Boletus scaber*). Plant catalases, however, are very unstable.

Catalase activity is determined by measuring the formation of oxygen gas by volume or by titrating the undecomposed H_2O_2 with $KMnO_4$ in H_2SO_4 solution or iodometrically. (See articles of various authors.)

Activity as Influenced by the Enzyme and Substrate Concentration

Von Euler and Josephson (3) express the activity of a catalase preparation as follows:

$$\text{Cat. f.} = \frac{\text{Reaction constant } k}{\text{Grams of enzyme in 50 cc.}}$$

(0.005–0.015 M H_2O_2, 0°, $M/150$ phosphate buffer of pH 6.8). k is the constant of the monomolecular reaction and a measure of the relative concentration of the enzyme in the digestion mixture (2).

Catalase action follows nearly a monomolecular reaction course. However, the constant often decreases as the time increases. Some of the enzyme is destroyed during catalysis. Greater concentrations of catalase require relatively less time than small concentrations (4, 5). Yamasaki (6) and Maximowitsch and Avtonomova (7) have independently given a mathematical expression of catalase action. They included H_2O_2 decomposition as well as enzyme destruction. This expression is according to Zeile (8) an advancement as compared with the empirical conception of the reaction course by Morgulis (9), who differentiates according to the given conditions between 1, $1\frac{1}{2}$, and 2 molecular reactions. For these mathematical considerations, a review on the kinetics of catalase by

Zeile (8) should be consulted. It should be noted, however, that the mathematical expression of Maximowitsch and Avtonomova (7) furnishes a theoretical basis for the change of the monomolecular reaction course to the bimolecular type by an increase of the substrate (as experimentally found by Morgulis) as well as the assumption that the total amount of H_2O_2 decomposed is a measure of the catalase concentration.

For the estimation of relative concentrations of enzymes, as for instance in studies of inhibitions, a graphic extrapolation of values for the monomolecular reaction constant *k*, until zero time, as applied by von Euler and Josephson (10), will suffice. Because of its simplicity, this method of elimination of enzyme decomposition by a series of experiments is at least as exact as the method of Yamasaki.

Optimum *p*H

Sörensen (11) found for liver catalase an optimum *p*H of 7.0. This was confirmed by Michaelis and Pechstein, by Morgulis and others. The catalase of leucocytes has also a *p*H optimum of 7.0 (12). Kidney (ox) catalase has an optimum between 6.8 (13), and malt catalase, 7.4 (14).

Williams (15) found liver catalase to have an optimum *p*H at 7.0, with a definite maximum stability towards H_2O_2 decomposition at this *p*H; i.e., the destruction constant is at a minimum. Nosaka (16), however, using blood catalase could not find any connection between optimum *p*H and enzyme destruction.

Schreus and Carrie (17) described a liver enzyme which has the ability to convert hematin to bilirubin. Protoporphyrin is an intermediary product. It is possible that this enzyme is identical with catalase.

Chemical Nature

It has been recognized by several workers that iron is an indispensable component of the active enzyme catalase

(18–21), and its relationship with hemin has been extensively studied. The catalytic activity of hemin is only one ten-thousandth that of the most active catalase preparation. Catalase is inhibited by HCN. Hemin, however, has a catalase activity only under certain definite conditions (22). Von Euler and Josephson (1) found highly active catalase preparations of horse liver to contain 0.6 per cent hemin. Zeile and Hellström believe that, by their chloroform method, hemoglobin may be completely separated from catalase, but that hemin, however, is an indispensable part of catalase. A catalase preparation obtained by this procedure gives an alkaline hematin spectrum, with a slight shift to the red part of the spectrum (about 10mμ).

With the addition of pyridine hydrosulfite and alkali, a typical hemochromogen spectrum is shown which is identical with that of blood hemin in every respect. The spectrum of the undenatured catalase indicates a special combination (Bindungszustand) of the hemin and the *colloidal system*. Spectroscopic experiments of Zeile and Hellström indicated a close relationship between the catalase hemin and protohemin or an isomer. The enzyme hemin was isolated in crystalline state by Stern (22*a*). By mixed melting point with synthetic mesoporphyrinester (after HJ–degradation) and by conversion into hemoglobin by coupling with globin it was shown to be protohemin and hence identical with the prosthetic group of hemoglobin. Purification methods have proved that the increase in enzyme activity is paralleled by increase in hemin content until a maximum activity of 40,000 cat. f. is reached. The relationship between activity and hemin content may be expressed by $\frac{k}{(\mathrm{Fe}p)}$, where k = monomolecular reaction constant of H_2O_2 decomposition and $(\mathrm{Fe}p)$ = number of milligrams of porphyrin iron per liter. $\frac{k}{(\mathrm{Fe}p)}$ was estimated to be 2500 in one preparation, whereas in another it was 3200.

The HCN inactivation is another method of showing that hemin is the active group of the enzyme catalase. If HCN is added to a concentrated enzyme solution in molar proportions, e.g., 1 mol HCN : 1 mol catalase hemin, there is a complete change in the original spectrum. The complex which forms is dissociable. The complex spectrum disappears on separation of the HCN by aeration.

Determinations of HCN inhibition, at varying catalase and HCN concentrations, harmonize with the dissociation equation of the mass action law (23, 24).

Stern (25) compared the inhibitory power of a number of sulfhydryl compounds (half maximum inhibition) to the inhibitory power of HCN:

HCN	6.3×10^{-6}	mol
Na_2S	8×10^{-6}	"
NaSH	3.2×10^{-5}	"
l-cystein	3.2×10^{-5}	"
SH-glutathione	5×10^{-3}	"

Phosphate also inhibits catalase (26).

Zeile and Hellström (2) found that the catalase prepared from sprouted squash seeds was identical with the animal catalase, since their absorption spectra, the complex spectra after the addition of HCN, and the HCN inhibition-dissociation constants ($K = 2.87 \times 10^{-7}$) are identical.

The catalase iron is present in a very stable ferric state. It cannot be reduced with hydrosulfite.

According to Michaelis and Pechstein (5) and Stern (26*a*), catalase is an ampholyte of a high molecular weight, with an isoelectric point of about *p*H 5.5. Waentig and Gierisch (27) found catalase to be a protein, digestible by trypsin. Similar results have been reported by Tauber and Kleiner (27*a*), who found that beef liver catalase is extremely sensitive to trypsin. Stern (28) estimated the diffusion velocity of catalase and reports that it is similar to that of hemoglobin.

The Alleged Reversible Hydrolysis of Liver Catalase. Agner (29) purified catalase from the horse liver by extracting the tissue with water, precipitation with ethyl alcohol-chloroform, adsorption on tricalcium phosphate, elution with secondary sodium phosphate, and dialysis. This final catalase preparation was hydrolyzed into two components by allowing it to dialyze against HCl. One component dialyzes through cellophane and is colored (hemin?), whereas the other one within the dialyzing bag is colorless and is a protein. The two components by themselves are inactive. If brought together, however, they very rapidly decompose H_2O_2; the hydrolysis of the catalase was carried out similarly to the hydrolysis of the oxidation ferment by Theorell. Tauber and Kleiner (27*a*) were not able to confirm the results of Agner, using the catalase of beef, rabbit, and rat liver, respectively.

That the hemin is accompanied by an indispensable protein has been noticed by all authors. No explanation has been offered as to why hemoglobin possesses only one ten-thousandth of the catalytic activity of catalase. Some investigators call the protein the "colloidal system" or "carrier" which is bound by a special combination to hemin. It appears now, however, that this combination is a chemical one, and the compound is probably a conjugated protein, or else an enzyme-coenzyme system.

Experimental Evidence for the Formation of an Enzyme-Substrate Compound

Catalase has been believed to be a classical example of an enzyme exhibiting an absolute specificity. It has been shown, however, by Stern (30) that, if concentrated catalase solutions are employed, monoethyl hydrogen peroxide is also attacked. By using this new substrate Stern obtained remarkable results which support the Michaelis-Menten theory. He furnished, the first time for any enzyme, experimental proof for the formation of an intermediary enzyme-substrate compound during the action of catalase on monoethyl hydrogen peroxide. By using a spectroscopic method he was able to analyze experimentally the two main phases of the enzyme reaction:

$$\text{Enzyme} + \text{Substrate} \rightleftharpoons \text{Enzyme-substrate compound} \quad (1)$$

$$\text{Enzyme-substrate compound} \rightarrow \text{Enzyme} + \text{Product molecules} \quad (2)$$

The first reaction he observed spectroscopically. "On direct visual observation in transmitted light and in the thickness of layer used for the spectroscopic experiments the enzyme solutions appear brown in color. Upon the addition of monoethyl hydrogen peroxide, there is a rapid change to a greenish hue. Within the following seconds the red color of the intermediate catalase-peroxide compound appears. In the course of the breakdown of the compound, which requires time of the order of minutes, the red tint fades and with the reformation of the free enzyme the original brown color is restored." Figure 22 represents a scheme of the corresponding changes in light absorption as seen with a spectroscope.

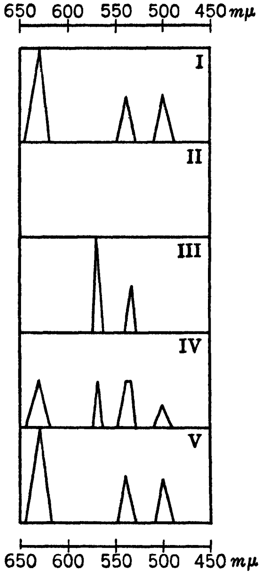


FIG. 22.—Schematic representation of the spectroscopic cycle. *I* represents the spectrum of free enzyme; *II*, lag period during which a greenish color but no discrete absorption bands are noticed; *III*, spectrum of enzyme-substrate compound; *IV*, coexisting intermediate and free enzyme; *V*, restored enzyme spectrum. (After direct observation with the spectroscope. The heights of the bands indicate their visual intensity)

The whole series of changes may be repeated by addition of more substrate. The original enzyme spectrum is restored by the disappearance of titratable peroxide from the system.

The over-all reaction (1 + 2) is determined by titration. The intermediate enzyme-substrate compound exhibits the properties postulated by Michaelis and Menten for an enzyme-substrate compound, and is not a mere adsorption complex, but a well-defined chemical compound, perhaps of the nature of a complex caused by the coordinative valences of the enzyme iron. The rate of formation of the intermediate enzyme-substrate compound is great compared with that of the complete reaction; and the temperature coefficient of its formation is smaller than that of the total reaction. The rate is not affected by the H ion concentration between *p*H 4 and 9.

Activity *pS* Curve for Catalase

The Michaelis constant (K_m) representing the affinity of catalase for monoethyl hydrogen peroxide has been determined by Stern (30) as shown in the activity *pS* curve in Fig. 23. K_m was found to be 0.02 M, which is of the same order as K_m of catalase for hydrogen peroxide (0.033 M).

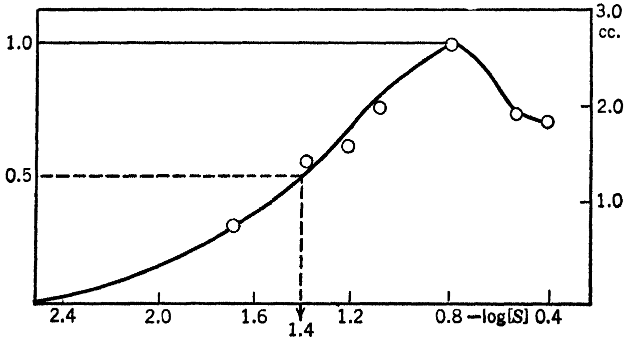


FIG. 23.—Activity-$P_{[S]}$ curve. The abscissa represents the negative logarithm of the substrate concentration; the right ordinate, amounts of peroxide decomposed in 5 minutes, expressed in cc. of 0.1 N thiosulfate; the left ordinate, the rational measure, the maximal reaction rate being taken as 1.0

REFERENCES

1. Euler, H. von, and Josephson, K.: Über Katalase. I. *Ann.*, **452**, 158 (1927).
2. Zeile, K.: Über die aktive Gruppe der Katalase. *Z. physiol. Chem.*, **195**, 39 (1931).
3. Euler, H. von, and Josephson, K.: Bezeichnung der Aktivität und Affinität von Enzymen. *Ber.*, **56**, 1749 (1923).
4. Senter, G.: Das Wasserstoffsuperoxyd-Zersetzende Enzym des Blutes. I. *Z. physik. Chem.*, **44**, 257 (1903); **51**, 673 (1905).
5. Michaelis, L., and Pechstein, H.: Untersuchungen über die Katalase der Leber. *Biochem. Z.*, **53**, 320 (1913).
6. Yamasaki, E. The chemical kinetics of catalase. *Sci. Rep. Tohoku*, **9**, 13, 75, 89 (1920).
7. Maximowitsch, S. M., and Avtonomova, E. S.: Über die Kinetik der Katalase. *Z. physiol. Chem.*, **174**, 233 (1928).
8. Zeile, K.: Katalase. *Ergebnisse Enzymforschung*, **3**, 265 (1934).
9. Morgulis, S.: A study of the catalase reaction. *J. Biol. Chem.*, **47**, 341 (1921).
10. Euler, H. von, and Josephson, K.: Über Katalase. II. *Ann.*, **455**, 1 (1927).
11. Sörensen, S. P. L.: Enzymstudien (Zweite Mitteilung. Über die Messung und die Bedeutung der Wasserstoffionenkonzentration bei enzymatischen Prozessen). *Biochem. Z.*, **21**, 131 (1909).
12. Stern, K. G.: Über die Katalase farbloser Blutzellen. *Z. physiol. Chem.*, **204**, 259 (1932).
13. Morgulis, S., Beber, M., and Rabkin, I.: Studies on the effect of temperature on the catalase reaction. I. Effect of different hydrogen peroxide concentrations. *J. Biol. Chem.*, **68**, 521 (1926).
14. Matsuyama, M.: Zur Kenntnis der Malzkatalase. *Biochem. Z.*, **213**, 123 (1929).
15. Williams, J.: Decomposition of hydrogen peroxide by liver catalase. *J. Gen. Physiol.*, **11**, 309 (1928).
16. Nosaka, K.: Studien über die katalytische Spaltung des Wasserstoffsuperoxyde durch das Blut (Erste Mitteilung. Über die chemische Dynamik der Blutkatalase). *J. Biochem.*, **8**, 275 (1928).
17. Schreus, H. T., and Carrie, C.: Untersuchungen zum Gallenfarbstoffwechsel. IV. Zur Natur des durch Eisessig-Äther-Extraktion nachgewiesenen Gallenfarbstoffs beim fermentativen Blutfarbstoffabbau in vitro. *Klin. Wochenschr.*, **2**, 1675 (1934).
18. Hennichs, S.: Zur Kenntnis der Katalase und ihrer Beziehung zu biologischen Oxydationen. *Biochem. Z.*, **171**, 314 (1926).
19. Warburg, O.: Atmungstheorie und Katalase. *Ber.*, **59**, 739 (1926).

20. Kuhn, R., and Wassermann, A.: Über die Abhängigkeit der katalitischen und peroxydatischen Wirkungen des Eisens von seiner Bindungsweise. *Ber.*, **61**, 1550 (1928).

20*a*. Kuhn, R., and Brann, L.: Über die katalytische Wirksamkeit verschiedener Blutfarbstoffderivate. *Z. physiol. Chem.*, **168**, 27 (1927).

21. Euler, H. von, and Josephson, K.: Über die katalytische Spaltung des Wasserstoffperoxyds durch Haemin. *Ann.*, **456**, 111 (1927).

22. Zeile, K.: Zur Kinetik der Hydroperoxydspaltung durch Porphyrin Metallkomplexsalze. *Z. physiol. Chem.*, **189**, 127 (1930).

22*a*. Stern, K. G.: The constitution of the prosthetic group of catalase. *J. Biol. Chem.*, **112**, 161 (1936).

23. Wieland, H.: Über den Mechanismus der Oxydationsvorgänge. IX. *Ann.*, **445**, 181 (1925).

24. Rona, P., and Damboviceanu, A.: Untersuchungen über die Leberkatalase. *Biochem. Z.*, **134**, 20 (1922).

25. Stern, K. G.: Über die Hemmungstypen und den Mechanismus der katalatischen Reaktion. *Z. physiol. Chem.*, **209**, 176 (1932).

26. Malkov, A. M.: Zur Frage nach der Rolle der Phosphate bei der alkoholischen Gärnung und Atmung der Hefe. *Biochem. Z.*, **262**, 185 (1933).

26*a*. Stern, K. G.: Der isoelektrische Punkt der Katalase. *Z. physiol. Chem.*, **208**, 86 (1932).

27. Waentig, P., and Gierisch, W.: Über die chemische Natur der Katalase. *Fermentforschung*, **1**, 165 (1914–16).

27*a*. Tauber, H., and Kleiner, I. S.: The chemical nature of catalase. *Proc. Soc. Exptl. Biol. Med.*, **33**, 391 (1935).

28. Stern, K. G.: Über die Teilchengrösse und das Molekulargewicht der Katalase. *Z. physiol. Chem.*, **217**, 237 (1933).

29. Agner, K.: Reversible Spaltung der Leberkatalase. *Z. physiol. Chem.*, **235**, **11** (1935).

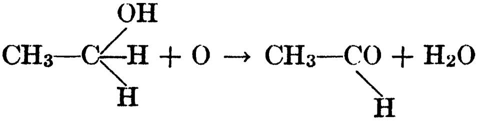
30. Stern, K. G.: On the mechanism of enzyme action. A study of the decomposition of monoethyl hydrogen peroxide by catalase and of an intermediate enzyme-substrate compound. *J. Biol. Chem.*, **114**, 473 (1936).

CHAPTER VII

OXIDIZING ENZYMES

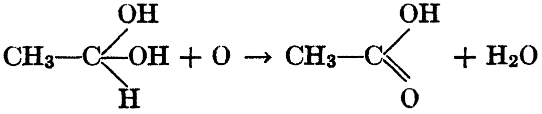
Biological oxidation has been explained in various ways; only two of the more important theories will be mentioned here. Some of these catalytic reactions are most interesting, for example, the enzymic oxidation of purine derivatives. These derivatives are among the most stable compounds known to the organic chemist. They are not readily oxidized by even concentrated HNO_3 and $KMnO_4$, and the recrystallization of uric acid from concentrated H_2SO_4 is a well-known laboratory experiment.

Wieland's Theory. According to Wieland (1), biological oxidation is based on removal of hydrogen by dehydrogenase from the substrate. For instance, when alcohol is oxidized to aldehyde by the cell, two hydrogen atoms are removed according to the equation



$$CH_3\text{—}\overset{OH}{\underset{H}{C}}\text{—}H + O \rightarrow CH_3\text{—}\underset{H}{CO} + H_2O$$

and when acids form from aldehydes it is also a dehydrogenation; i.e., acids form from aldehyde hydrates according to the equation



$$CH_3\text{—}\overset{OH}{\underset{H}{C}}\text{—}OH + O \rightarrow CH_3\text{—}\overset{OH}{\underset{O}{C}} + H_2O$$

The theory of Wieland is supported by the fact (as will be seen below) that oxygen is not necessary for oxidation. It may be replaced by another hydrogen acceptor, as for instance a reducible dye.

Warburg's Theory. Warburg's theory (2), which is also supported by experiments, is contrary to Wieland's contention. According to Warburg the cell catalysts (hemins) contain iron which is able to activate molecular oxygen. According to this author the "respiratory ferment" ($X \cdot Fe$) combines with the oxygen in the following way: $X \cdot Fe + O_2 = X \cdot FeO_2$. The oxidized iron substance is able to oxidize organic compounds according to the equation: $X \cdot FeO_2 + 2A = X \cdot Fe + 2AO$. Warburg has shown that substances like HCN, which are able to form with iron non-catalytic complexes, inhibit oxidation (3).

Wieland's standpoint was greatly supported by the introduction of the methylene blue technic by Thunberg (4). This method permits the oxidation of metabolites under anaerobic conditions, methylene blue playing the rôle of a hydrogen acceptor, the metabolite being the hydrogen donator. The methylene blue becomes reduced to an almost colorless leuco compound. Thunberg tested a great number of substances of which many were able to become oxidized by the dye in the presence of fresh tissue. The catalytic effect disappeared on heating the tissues.

Keilin, whose work will be discussed, found that the pigment cytochrome forms a link between the hydrogen-activating and oxygen-activating system of the cell. The oxidized cytochrome is reduced by the dehydrogenases and their substrates. The reduced pigment is reoxidized by combining with oxygen through the action of an oxygen-activating enzyme, indophenol oxidase or cytochrome oxidase.

(A) DEHYDROGENASES OR ANAEROBIC OXIDASES

Harrison (5) defines dehydrogenases as "tissue enzymes responsible for activation of the molecules of the metabolites so that they can be oxidized in the presence of oxygen or a suitable reducible substance." They have also been called anaerobic oxidases, dehydrases, and oxidoreductases.

Dehydrogenases convert non-reducing substances into compounds of high reducing power.

Succinic Dehydrogenase

This is the best-known enzyme of this group, and it is found in all tissues. It is insoluble in H_2O but soluble in a slightly alkaline medium and in dilute salt solutions. Ohlsson (6) obtained the enzyme by mincing horse meat, washing it with water, and extracting it with $M/15$ Na_2HPO_4 of pH 9.0. The centrifuged fluid contains the enzyme. Others employed different methods (7). This extract contains another enzyme, fumarase, which may be removed by warming the washed tissue at 50° before extracting with phosphate. Succinic dehydrogenase converts succinic acid into fumaric acid. It was first discovered by Einbeck (8). This enzyme requires oxygen, or in the absence of oxygen, methylene blue, for its action. Aerobic oxidation is inhibited by HCN (Batteli and Stern), but not anerobic-methylene blue oxidation (Thunberg). These findings led to the conclusion of Fleisch (9) and of von Szent-Györgyi (9*a*) that both hydrogen activation and oxygen activation may take place in biological oxidations. In the presence of HCN, which inhibits the iron-containing (Warburg) oxygen activator, the oxygen uptake of succinic acid is prevented. The reduction of the methylene blue, however, can take place by the hydrogen-activating fraction of the enzyme, which does not require oxygen and is not inhibited by HCN.

According to Keilin the succinic enzyme is a complete enzyme system consisting of a dehydrogenase, cytochrome, and the oxygen-activating indophenol (or cytochrome) oxidase. The cytochrome is alternately reduced by the dehydrogenase with succinic acid and oxidized by the oxidase with oxygen. After HCN inhibition the indophenol oxidase can reduce methylene blue but not oxygen. The aerobic activity may be separated from the anaerobic

(10, 11). Dixon (12) does not believe that indophenol oxidase is part of the succinic enzyme (see also 13). Euler and associates (14) found that succinic enzyme preparations lose their aerobic activity on dialysis, but not the anaerobic one.

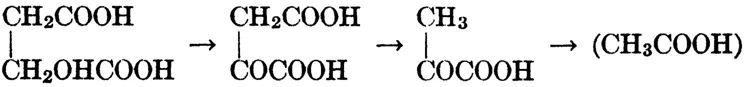
Quastel and Whetham (15) made an interesting experiment. They found that when succinic acid was added to *resting* bacteria it reduced methylene blue and the succinic acid was changed to fumaric acid. By starting with the fumaric acid and leuco methylene blue they were able to reverse the reaction. Thunberg (16) carried out this same experiment, using muscle dehydrogenase.

Sen (17) found that narcotics inhibit this enzyme.

Malic Dehydrogenase

Malic dehydrogenase may be prepared by washing frog or ox muscle with $M/15$ phosphate buffer of pH 6.6. The washed tissue reduces methylene blue and also takes up oxygen. Here, apparently, an oxygen-activating mechanism is linked up with the dehydrogenase (18).

According to Hahn (19), malic acid is first oxidized by the muscle enzyme to oxaloacetic acid, which is decarboxylated to pyruvic acid:



$$\begin{array}{l}\mathrm{CH_2COOH}\\ |\\ \mathrm{CH_2OHCOOH}\end{array} \rightarrow \begin{array}{l}\mathrm{CH_2COOH}\\ |\\ \mathrm{COCOOH}\end{array} \rightarrow \begin{array}{l}\mathrm{CH_3}\\ |\\ \mathrm{COCOOH}\end{array} \rightarrow (\mathrm{CH_3COOH})$$

The natural *l*-malic acid is dehydrogenated much faster than the dextro form.

Lactic Dehydrogenase

Lactic dehydrogenase, because of the importance of lactic acid in metabolism, has been extensively studied. It oxidizes lactic acid to pyruvic acid

$$\mathrm{CH_3CHOHCOOH{—}2H} \rightarrow \mathrm{CH_3COCOOH}$$

Oxidation by this enzyme requires a coenzyme. The coenzyme is easily extracted from the tissue by washing with H_2O; the lactic acid dehydrogenase is not. Extracting the washed tissues yielded inactive tissues as well as inactive extracts. This is the main reason that the chemistry of this enzyme developed only slowly. Meyerhof (20) found that aerobic oxidation of lactic acid in the presence of washed muscle was stimulated by the addition of boiled H_2O extracts of animal tissues and of yeast. Von Szent-Györgyi (21) believes this coenzyme to be identical with cozymase.

Although lactic acid dehydrogenase is present in a great many tissues, cell-free preparations have only recently been studied. Banga, von Szent-Györgyi, and Vargha (22) use the following method: The muscle of the pig heart is chopped and washed twice for ten minutes with twenty times its weight of distilled water. The residue is squeezed out and frozen. The frozen tissue is ground and extracted with three times its original weight of $M/15$ phosphate buffer of *p*H 7.2. Boyland (23) and Holmberg (24) have also obtained very active cell-free preparations, and Boyland and Boyland (25) have more recently isolated a soluble lactic enzyme from heart muscle which can also oxidize malic acid (see also Birch and Mann [26]). Gözsy and von Szent-Györgyi (27) reported an inactivation of their lactic enzyme by methylene blue in the presence of oxygen and lactate.

The nature of the lactic coenzyme has been extensively studied recently in von Szent-Györgyi's laboratory (28–31). The coenzyme has been obtained as a crystalline picrate, and was found to be an adenyl nucleotide. This coenzyme can replace cozymase in glucose fermentation (32).

β-Hydroxybutyric Dehydrogenase

Wishart (33) has shown that liver (Na_2HPO_4) extracts are able to reduce methylene blue in the presence of β-hydroxybutyric acid. Harrison and Thurlow (34) believe that not enough evidence is available to justify the existence of a specific β-hydroxybutyric dehydrogenase, and Banga, Laki, and von Szent-Györgyi (35) found that the oxidation of β-hydroxybutyric acid to acetoacidic acid is due to the same enzyme-coenzyme system which oxidizes lactic acid.

Citric Dehydrogenase

Citric dehydrogenase oxidizes citric acid probably to acetone dicarboxylic acid. First two hydrogen atoms are given off, followed by the loss of CO_2.

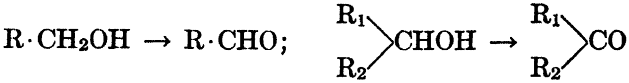
This reaction was first observed by Thunberg (36) and has been studied since by others (37, 38). Bernheim (39) prepared the cell-free enzyme by the following procedure:

Ground pig, ox, or sheep liver is extracted with acetone. The extracted liver tissue is dried *in vacuo* and extracted with H_2O. The extract is dialyzed and centrifuged, and the clear liquid, which still contains some hemoglobin, is ready for use. This preparation, according to Bernheim, is very specific, since it did not oxidize any other substrate. Harrison (40) found it to be active toward hexosediphosphoric acid. Andersen (41) believes that cozymase may act as a coenzyme for the oxidation of citric acid by citric acid dehydrogenase.

Alcohol Dehydrogenase

Batteli and Stern (42) were the first to study the oxidation of alcohol by the tissues of various animals. They found that liver and kidneys are the only sources of this enzyme. Active preparations have been obtained by Wieland and Frage (43) and by Mizusawa (44), who found inhibition with KCN, pyrol, adrenalin, and ultra-violet rays. Lehmann (45) states that on the addition of Fiske's adenosine triphosphate the activity of horse muscle alcohol dehydrogenase can be greatly increased.

Alcohol dehydrogenase converts ethyl alcohol into acetaldehyde and isopropyl alcohol into acetone. The enzyme simply dehydrogenates primary alcohols to aldehydes and secondary alcohols to the corresponding ketones.

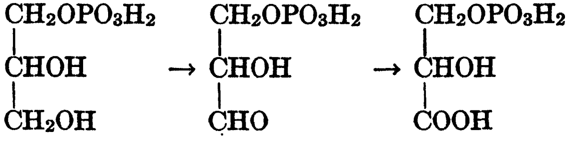


$$R\cdot CH_2OH \rightarrow R\cdot CHO; \quad \begin{matrix} R_1 \\ R_2 \end{matrix}\!\!>\!CHOH \rightarrow \begin{matrix} R_1 \\ R_2 \end{matrix}\!\!>\!CO$$

This enzyme differs from the aldehyde-oxidizing *Schardinger* enzyme (of milk) in that it oxidizes alcohol. Good hydrogen acceptors for this enzyme are oxygen, methylene blue, and quinone. HCN inhibits the enzyme's oxygen uptake. Müller, however, reports that he obtained alcohol dehydrogenes from yeast, which was not affected by HCN (46). Euler and Adler (46*a*) believe that the HCN stability of the yeast enzyme is due to the flavine enzyme.

Glycerophosphoric Dehydrogenase

This enzyme has been obtained recently by Lehmann (47) from yeast and does not contain other, related enzymes. The activation of glycerophosphoric dehydrogenase is accelerated by adenosine triphosphate. The α-glyceromonophosphoric acid is converted into the corresponding glyceric aldehydephosphoric acid, which is further changed into glyceric acid monophosphoric acid ("phosphoglyceric acid"):



$$\begin{matrix} CH_2OPO_3H_2 \\ | \\ CHOH \\ | \\ CH_2OH \end{matrix} \rightarrow \begin{matrix} CH_2OPO_3H_2 \\ | \\ CHOH \\ | \\ CHO \end{matrix} \rightarrow \begin{matrix} CH_2OPO_3H_2 \\ | \\ CHOH \\ | \\ COOH \end{matrix}$$

It is not known whether both oxidations are carried out by one or more enzymes. According to Davies and Quastel this enzyme is very resistant toward narcotics and barbituric acid derivatives (48).

Hexosediphosphoric Dehydrogenase

Hexosediphosphoric dehydrogenase was first noticed by Broman (49) in muscle extracts. Thunberg found it in extracts of cucumber seeds (50). Harrison's (40) experiments show that the oxidation of fructose diphosphate can be brought about in the presence of methylene blue. His experiments indicate that the reaction is due to two separate enzymes. The enzyme preparations of ox liver and of cucumber seed behaved similarly. A coenzyme is necessary for this reaction (51).

Schardinger Aldehyde Dehydrogenase and Xanthine Dehydrogenase

The reduction of methylene blue by aldehydes in the presence of milk was first noticed by Schardinger in 1902 (52). Twenty years later Morgan, Stewart, and Hopkins (53) showed that hypoxanthine and xanthine are also able to reduce methylene blue in the presence of milk. They found that this reaction can be brought about by various animal tissues and that it is due to the known action of the xanthine "oxidase" which oxidizes hypoxanthine and xanthine to uric acid. It is not known whether the aldehydes and purines are oxidized by one and the same enzyme. The reaction takes place when original unboiled milk is used, which is a good source of this enzyme or enzymes and other dehydrogenases are few and present in milk only in traces. Extensive studies on liver xanthine and aldehyde dehydrogenase have been published by Morgan (54).

Aldehydes are converted by the dehydrogenase (*a*) into saturated fatty acid and (*b*) by a mutase reaction (Cannizzaro) in which one molecule of aldehyde is oxidized to the acid and another molecule of aldehyde is reduced to the alcohol. The reactions proceed simultaneously:

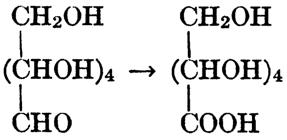
(*a*) $R \cdot OH$
(*b*) $2RCHO + H_2O \rightarrow RCH_2OH + RCOOH$

A series of papers have been published recently dealing with the identity of the two enzymes. The results are conflicting (55–57).

Glucose Dehydrogenase

Glucose + dehydrogenase was first obtained by Harrison (58) from liver extracts. According to Mann (59), the enzyme requires an activator or coenzyme. This activator is also present in the liver. With glucose it reduces methylene blue slowly, which is probably due to the presence of traces of the coenzyme. On the addition of the coenzyme, however, the dye is rapidly reduced. The glucose dehydrogenase (enzyme-coenzyme), when prepared by the method of Harrison, contains also a *triose dehydrogenase* (57).

Glucose + dehydrogenase does not combine with oxygen unless methylene blue is added. The reduced leuco compound is then rapidly reoxidized by air oxygen, functioning as a hydrogen carrier. The dehydrogenase system converts *d*-glucose into *d*-gluconic acid:



$$\begin{array}{ccc} CH_2OH & & CH_2OH \\ | & & | \\ (CHOH)_4 & \rightarrow & (CHOH)_4 \\ | & & | \\ CHO & & COOH \end{array}$$

Other sugars are not affected by this enzyme. The Schardinger enzyme does not oxidize glucose. This catalysis may be carried out in the presence of several oxidizing agents. The dehydrogenase may be prepared from bacteria and molds (60).

A simple method for the preparation of the enzyme and coenzyme has been given by Harrison (61).

Other interesting studies concerning this enzyme have been published recently by Lundsgaard (62), Meyerhof (63) and Harrison (64). Harrison also found that the lactic coenzyme can function as the coenzyme of glucose dehydrogenase. The glucose coenzyme, however, could not replace the lactic coenzyme (65). It has been shown that the glucose dehydrogenase coenzyme is identical with cozymase (66). Mann (59) showed that glutathione may be reduced by glucose dehydrogenase in the presence of glucose.

Amino Acid Dehydrogenases

Glutamic acid dehydrogenase of muscle and liver oxidizes glutamic acid in the presence of methylene blue (67). This is a well-defined enzyme. No ammonia or urea is liberated, however (68, 69). Krebs (70) detected α-ketoglutaric acid as an oxidation product of glutamic acid.

Bernheim and Bernheim (71) obtained from the liver an enzyme preparation which is able to oxidize proline and hydroxyproline. The same authors showed that broken cell suspension of the liver and kidney can oxidize alanine (72). Both are aerobic enzymes, and they are not inhibited by HCN. They act, however, also in the absence of oxygen if methylene blue is added as a hydrogen acceptor. These enzymes are rare examples of the cyanide-resistant, anaerobic-aerobic type.

The Methylene Blue Technic of Thunberg and Ahlgren (72*a*) for Study of Dehydrogenases (Anaerobic Oxidases)

The method is based on the following principle: The enzyme material is placed in a solution containing methylene blue, phosphate buffer, and the substrate. The tube is evacuated and placed in a water bath of constant temperature. The time necessary for the methylene blue to be decolorized is estimated. Thus the rate of oxidation in the mixture can be measured, and the nature of the oxidizable substrate and the factors influencing its oxidation can be studied.

Method. The vacuum tube as suggested by Thunberg (Fig. 24) is most convenient. It should be of hard, colorless glass and of about 10-cc. capacity. Into each of three tubes 0.9 cc. of a mixture of 8 cc. of methylene blue 1 : 2000 (stock solution of methylene blue should be made by dissolving 1 gram in 500 cc. of water) and 6 cc. $M/5$ phosphate buffer of *p*H 7.20 is placed. (The *p*H of the buffer and the concentration of the methylene blue may be varied if many tubes are available.) Then 0.1 cc. water is added to the first tube, 0.1 cc. of 0.1 *M* potassium succinate to the second, and 0.1 cc. of 0.1 *M* potassium glycerophosphate to the third. All solutions should be of the same temperature. Now 0.2 gram of finely divided rabbit or other animal muscle, which has been freed of ligament and fat, and washed until free of blood, is added. A measured amount of tissue extract may be used instead of the tissue. Evacuate tubes until pressure is less than 10 mm. Foaming may be stopped by rotating the tubes in a horizontal position.

These examples show the action of succinic dehydrogenase (second tube) converting succinic acid into fumaric acid; and the action of glycerophosphoric dehydrogenase (third tube) converting α-glyceromonophosphoric acid into the corresponding glyceric aldehydphosphoric acid, which is further changed to glyceric acid monophosphoric acid.

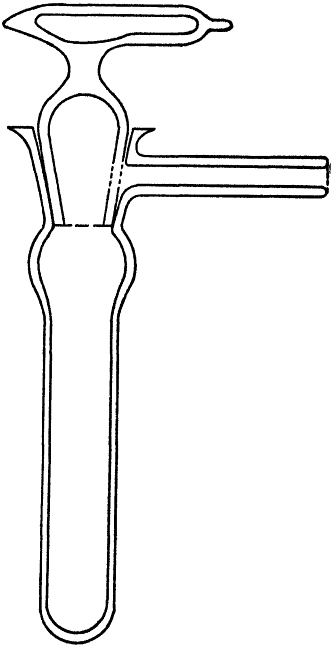


FIG. 24.—Thunberg's Vacuum Tube

The substrates of dehydrogenases are oxidized in the presence as well as in the absence of oxygen. In the absence of oxygen, however, a *hydrogen acceptor* such as methylene blue is needed:

$$M \text{ (methylene blue)} + 2\text{H} \rightarrow M\text{H}_2 \text{ (methylene white)}$$

Similar reactions take place in the living tissues (*see Some of the Functions of Citochrome in Relation to Oxidases*).

Thunberg's method has been modified by Green and Dixon (72*b*). Their modification is more suitable than the original method, when extreme accuracy is desired.

Detailed methods for the study of oxidases have been described by Dixon (72*c*) in an inexpensive but very practical laboratory handbook.

(B) OXIDASES OR AEROBIC OXIDASES

The following part of this chapter will deal with enzymes which oxidize only in the presence of hydrogen acceptors. They may be called aerobic oxidases or simply oxidases. The dehydrogenases or anaerobic oxidases have been discussed in the preceding pages. It has been shown that dehydrogenases activate hydrogen in the substrate on which they act. By this they convert non-reducing substances into reducing substances (succinic acid, malic acid, lactic acid, β-hydroxybutyric acid, citric acid, etc.). *In vitro* strong oxidizing agents would be required to oxidize these compounds. The oxidases or aerobic oxidases, however, oxidize substances which have already some reducing power and which are slowly oxidized by air without the aid of a catalyst. Typical examples are ascorbic acid and phenols. The hydrogen in them is already in an active form. A small increase in oxidation-reduction potential accelerates their rate of oxidation greatly. Oxidation may be brought about by a further activation of hydrogen, i.e., by colloidal palladium or an oxidase.

Catalases, peroxidases, and ascorbic acid oxidase are poisoned by HCN but not by CO. Some activators of molecular oxygen, however, are very sensitive to CO.

Peroxidases—Their Importance as Biological Oxidants

Peroxidases are constituents of many tissues, especially good sources being the spleen and lung (73*a*). The roots and seedling sprouts of higher plants contain large quantities; the best source is horseradish (73). The biological function of peroxidases is to transfer peroxide oxygen to oxidizable substances. Unlike the catalases, peroxidases do not decompose H_2O_2 in the absence of an oxidizable substrate. Dakin showed (as early as 1905) (74) that many products of intermediary metabolism can be oxidized by H_2O_2 and that sometimes iron is required for the oxidation. Reduced glutathione may also be oxidized by H_2O_2. Keilin (75) showed that another cell constituent—cytochrome *c*—is easily oxidized by H_2O_2. That H_2O_2 is formed in the living cell has never been demonstrated. It has been shown, however, that a number of dehydrogenases (xanthin dehydrogenase, aldehyde dehydrogenase, succinic dehydrogenase) as well as cysteine and glutathione do form H_2O_2 *in vitro,* during aerobic oxidation (76–79). There is a formation of H_2O_2 in bacterial metabolism (80, 81). Catalase, a constituent of all tissues, would also decompose an excess of the toxic H_2O_2.

These facts indicate that H_2O_2 is probably a normal tissue constituent. The work of a number of authors suggests that H_2O_2 might be an important biological oxidizing agent (76, 82, 83, 84). It should be noted that the important metabolites such as glucose, glycerol, glycine, glutamic acid, phenylalanine, and many related substances are not oxidized by peroxide-peroxidase (83). The action of peroxidase in the living tissue is not known.

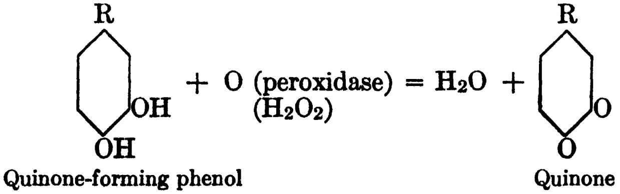
Color Reactions

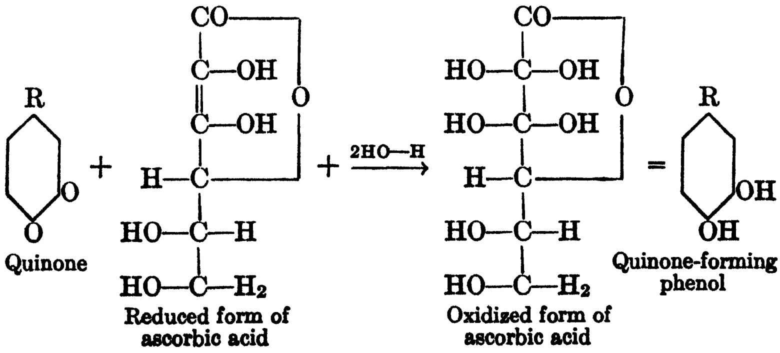
Bach and Chodat (85) found that potato scrapings, when mixed with gum guaiac or guaiaconic acid, turn blue, and that the color is due to the oxidation of guaiaconic acid by active oxygen. Extracts of the roots of horseradish did not give this test, but when H_2O_2 was added, the blue color appeared. No oxygen was liberated when H_2O_2 was added alone, nor did any other change occur. Since the effect disappeared when the horseradish extract was boiled, Bach and Chodat attributed it to an enzyme which they called peroxidase (86–88). Later it was found that substances like oxyhemoglobin and hemocyanin (86–88), and inorganic salts like ferrous sulfate and the chlorides of cobalt, copper, and nickel, may also give the guaiac test (89). Benzidine, guaiacol, *o*-phenylenediamine, quinol, phenolphthalein, pyrogallol, a mixture of *p*-phenylenediamine and α-naphthol, as well as other organic substances, have been used for the detection of peroxidases. These reagents, however, are not specific for peroxidases. *o*-, *m*-, and *p*-cresol have been recommended for the identification of this enzyme (90, 91). A 0.1 per cent solution of *o*-cresol, with peroxidase in the presence of H_2O_2, produces a green to brown color; laccase gives the same color without the H_2O_2. Tyrosinase has no effect on *o*-cresol. Guaiacol may be used instead of *o*-cresol for the differentiation of these enzymes. The nature of the substances produced from cresols is not known. The oxidation products of other compounds, however, are known. Quinol produces *p*-benzoquinone, which, with an excess of quinol, forms quinhydrone. Pyrogallol changes to purpurogalin (92). Willstätter has shown that peroxidase forms a very active compound with H_2O_2 (92*a*).

Interaction of Ascorbic Acid and Peroxidase (93)

Undialyzed peroxidase preparations and peroxide oxidize ascorbic acid with great rapidity; dialyzed solutions, however, do not act on the vitamin. In the undialyzed peroxidase solution certain substances are present which form quinones with peroxide-peroxidase. The quinones oxidize the ascorbic acid, and they are in turn reoxidized by peroxide-peroxidase. The oxidation of ascorbic acid and reduction of quinones proceeds until all the peroxide is reduced, resulting in the decomposition of the physiologically toxic peroxide. The oxidized ascorbic is readily reduced again by glutathione (93*a*). In the following two typical experiments will be described:

Oxidation of Ascorbic Acid by Dialyzed Peroxidase and Quinone-forming Compounds. *Vanillin.* After three days of dialysis of the peroxidase solution against 2 liters of water which has been frequently changed, its ability to oxidize ascorbic acid is completely lost but its leuco-malachite green oxidizing power is not affected. On the addition of a small amount of vanillin, the solution becomes active again and even surpasses the original activity many times. There is a rapid increase of activity when 2 to 9 mg. of vanillin is added. Above 9 mg., however, the increase remains almost stationary when the vanillin is added to 2 mg. of ascorbic acid, 3 cc. of buffer (borax 0.05 *M* – KH_2PO_4 0.1 *M*) of *p*H 5.8, 1 cc. of 0.02 *M* H_2O_2, and 0.5 cc. of dialyzed peroxidase solution. Total volume 12 cc., temperature 20°, time 5 minutes. This reaction with vanillin shows two peaks, one at *p*H 5.8 and one at *p*H 9.0. At *p*H 9.0 there is maximum activity. No colored compound is formed during this reaction. (See general Scheme.)





Oxidation of Ascorbic Acid by Dialyzed Peroxidase and a Cold Extract of Suprarenal Glands. To avoid oxidation of possible reducing substances in the active suprarenal gland, an extract was prepared as follows:

Beef suprarenal glands were frozen with "dry ice" at the slaughter house immediately after removal. To 70 grams of the frozen glands which were ground in a meat chopper, 100 cc. of 0.1 *N* HCl and 200 cc. of triple distilled water were added and shaken in an Erlenmeyer flask for five minutes. The extract was then centrifuged in a dry ice-cooled 250-cc. centrifuge flask and the supernatant fluid was filtered in a refrigerator. The clear extract which had a *p*H of 5.0 was very active at *p*H 5.8 (borax-phosphate) but less active below and above this *p*H. For instance, when to 2 mg. of ascorbic acid (in 3 cc. of water) 4 cc. of a buffer of *p*H 5.8, 2 cc. of 0.02 *M* H_2O_2, 0.5 cc. of dialyzed peroxidase, and 5 cc. of suprarenal extract were added, in five minutes at 20°, 75 per cent of the ascorbic acid was oxidized. At *p*H 7.0 and 3.6 respectively, only 20 per cent of the ascorbic acid was oxidized under similar conditions and at *p*H 7.8 none. The same experiments with 1 to 15 mg. commercial adrenaline showed only slight activity.

This shows that, besides adrenaline, certain powerful quinone-forming reducing substances must be present in the active suprerenal gland.

The optimum conditions of this reaction, such as velocity and optimum *p*H, are governed by the nature of the quinone-forming compound (93). It should be noted that ascorbic acid oxidase, which is very specific since it acts only on vitamin C, does not require peroxide or any other compound for its activity (137). Ascorbic acid oxidase, however, is not present in animal tissues.

Preparation

Willstätter and associates have described several methods for the preparation of a number of plant peroxidases

(94). Elliott (83) obtained a very active peroxidase, free from catalase, by fractional precipitation with ammonium sulfate from milk. For a practical method for the preparation of horseradish peroxidase see Ref. 93.

Estimation

The color produced from pyrogallol is determined colorimetrically, or the ether-soluble purpurogalin is weighed (95, 96). The colorimetric method of Willstätter and Weber (96*a*), by which the oxidation of leucomalachite green is followed, is very convenient for plant peroxidase estimation. A microgasometric method has recently been described (83).

Effect of *p*H

Horseradish peroxidase has an optimum *p*H at 4.5 to 6.5 when guaiacol is the substrate. With *o*-cresol it is at 3.5 to 5, and with pyrogallol the optimum cannot be determined, since the polyphenol formed is autoxydizable (97). Elliott (83) showed that milk peroxidase is active over a wide range on the *p*H scale. In an alkaline medium from *p*H 8 to nearly 10, the activity decreases but recovers immediately on neutralization. At *p*H 10 practically all the enzyme is destroyed. In acid medium at *p*H 4.2 to 3.8 a precipitate is formed and a decrease in activity takes place; between *p*H 3.6 and 3.2 the precipitate redissolves and the enzyme is completely inactivated. On adjustment to about *p*H 7 and standing over night the peroxidase is wholly active again. This is a highly interesting phenomenon, worth further consideration.

Chemical Nature

It has been stated that peroxidase is a heme compound (98). This, however, has been contradicted (99).

Sumner and Howell (99*a*) obtained a highly active peroxidase from fig sap. It contained hematin, of which all was in the combined form. These authors believe that "the purified peroxidase may have contained a reduced hematin compound."

Tyrosinase or Monophenol Oxidase

Tyrosinase was discovered by Bourquelot and Bertrand (100) in the fungus *Russula nigricans.* Bertrand (101) found that this enzyme oxidizes the amino acid tyrosine to a black pigment (melanin) and that peroxidase and laccase are not able to do this. Tyrosine has since been found to be very abundant in the plant kingdom and the tissues of invertebrates. The enzyme occurs often with tyrosine, which is the reason for the darkening of the cut surface of many plants and vegetables. Several years later Bertrand (102) reported that tyrosinase can oxidize phenols besides tyrosine. The phenols may be used to differentiate between tyrosinase and laccase (tyrosine, *p*-cresol, and guaiacol). *Tyrosine* plus tyrosinase plus guaiacol gives a red color which later turns dark brown. Laccase does not affect tyrosine. *p*-Cresol plus tyrosine produces an orange color (103). *Laccase* gives a milky suspension with *p*-cresol. *Guaiacol* plus tyrosinase has no effect, but laccase produces a brown-red color due to tetra-guaiacol formation.

Preparation. Tyrosinase may be prepared from potato peelings, wheat bran, fungi (*Lactarius, Russula*), and the mealworm (*Tenebrio molitor*). Chodat and Staub (104) made laccase and peroxidase preparations, extracting potato peelings with H_2O and precipitating the enzyme by adding alcohol to make the aqueous solution 40 per cent alcoholic. The precipitate was then dissolved in H_2O and precipitated with alcohol. By repeating the precipitation the preparation was made free of laccase and peroxidase. It is easier to obtain such a preparation from the mealworm. The worms are rubbed with chloroform water and the hulls removed by filtration through muslin. The milky suspension is now filtered through paper and washed with chloroform water containing a trace of acetic acid until the washings give only a small precipitate with lead acetate. The precipitate is rubbed with chloroform water and made slightly alkaline with ammonium hydroxide. These preparations keep well for months if preserved with chloroform. The extract may be centrifuged before use.

Optimum *p*H. Tyrosinase is most active between *p*H 6 and 8. It is active between *p*H 5 and 10. In alkaline solution it is quite stable but not in acid medium (Raper).

The Effect on Phenols. The earlier view was that tyrosinase deaminizes tyrosine. Later it was found that phenols are necessary for this reaction. Happold and Raper (105) repeated the earlier experiment and found no deaminizing action on amino acids unless *p*-cresol was present. This was confirmed by others (106). Raper and Wormal showed that tyrosinase does not liberate NH_3 from tyrosine and that the black pigment melanin contains more nitrogen than tyrosine. Happold and Raper, and von Szent-Györgyi (107), reported independently that *o*-quinones are produced by the action of tyrosinase upon phenol, *m*-cresol, *p*-cresol, catechol, and homocatechol. *o*-Quinones, however, are not the final products (Robison and McCance, and Pugh and Raper) (108). For a further discussion of the action of tyrosinase and the formation of melanin, see the recent excellent review by Raper (109).

Dopa Oxidase

Dopa oxidase (110) is the enzyme which oxidizes 3 : 4-dihydroxyphenylalanine ("dopa") to melanin. It is found in the melanoblast of the epidermis. It may be detected by fixing skin sections in formaldehyde and treating them for a time varying from a few hours up to a few days with a 0.1 per cent solution of pure 3 : 4-dihydroxyphenylalanine buffered to *p*H 7.3 to 7.4. After keeping the tissue twenty-four hours or so in the reagent, the cells containing the oxidase will be deeply stained with melanin. Only the pigment-forming cells (melanoblasts) give this color reaction. Methods for the preparation of this enzyme have been given by Bloch and Schaaf (111) and by Albl (112). Dopa oxidase, according to Bloch and Schaaf, does not give color reactions with monophenolic substances, with catechol derivatives, or with polyphenolic compounds. According to recent findings the dopa oxidase is stereo specific. It reacts only with the natural *l*-substrate (113-115). More recently, however, the specificity of dopa oxidase has been seriously questioned (116). There is no doubt that tyrosinase and laccase are closely related to dopa oxidase.

Laccase or Polyphenol Oxidase

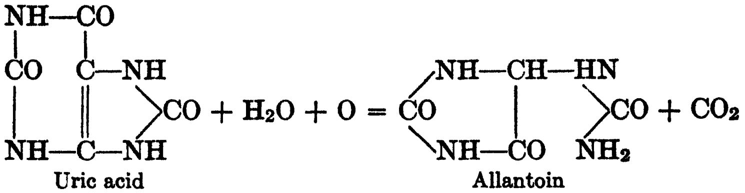
This type of enzyme oxidizes polyphenols such as *o*- and *p*-, di- and triphenol with the formation of the respective quinones as primary products. Tyrosins, monophenols, and aromatic diamins are not attacked. Laccase, like tyrosinase and peroxidase, forms purpurogallin from pyrogallol. This reaction is used for the quantitative estimation of this enzyme too. Laccase is found in various bacteria (117). In mushrooms the darkening of the cut surface is due to the action of this enzyme (*Lactarius, Russula, Boletus, Agaricus* are examples) (118). Mushrooms do not contain peroxidases, which simplifies the study of laccases in this material (119). Many higher plants and fruits contain laccase (120). It is interesting to note that there is no laccase in citrous fruit. Laccase is present in the leucocytes of invertebrates (121) and vertebrates (122). It has been found in egg white of the hen (123). This enzyme has an optimum from pH 6.7 to 8, depending upon the substrate (guaiacol) concentration (124). HCN inhibits laccase action greatly (125).

Indophenol Oxidase

The indophenol color reaction was first noticed by Ehrlich (126) in 1885, when he injected dimethyl *p*-phenylenediamine and *α*-naphthol into animal tissues, which turned blue (indophenol). This is called the "Nadireaction," the term being derived from the first two letters of the name of the reagents used. In 1896 Pohl (127) found that plant tissues are also able to give the indophenol reaction. Batelli and Stern (128) studied the quantitative distribution of this enzyme; i.e., they measured the O_2 uptake when *p*-phenylenediamine was added to various tissues. They found brain tissue to be most active, whereas blood cells, heart, muscle, kidney, and liver were fairly rich in this activity. According to Keilin (129), CO and HCN inhibit yeast and mammalian muscle indophenol oxidase. It does not attack polyphenols (130). He is of the opinion that this enzyme forms with cytochrome an intracellular catalytic system which is probably Warburg's oxidation system of the cell (131).

Uricase

Uricase oxidizes uric acid to allantoin and carbon dioxide:



The enzyme may be prepared from liver, kidney, and cattle brains (132). It has been suggested that hydroxyacetylene-diureincarbonic acid is an intermediary product in the formation of allantoin from uric acid by uricase, and that the process is similar to that of MnO_2 oxidation (133).

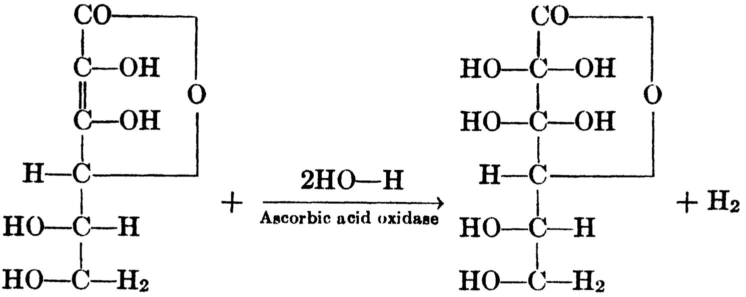
Ascorbic Acid (Vitamin C) Oxidase

Von Szent-Györgyi (134) noticed that if a part of a cabbage leaf is put in a respirometer in the presence of KOH it takes up oxygen. Mincing decreased this activity of the leaves. When ascorbic acid was added to the pulp, it became oxidized. Boiled pulp did not show this effect, hence he concluded it to be an enzymic reaction. Von Szent-Györgyi (135) believed, however, that this enzymic function is a complicated one, and formulated a theory by which he tried to explain the indirect oxidation of ascorbic acid (at that time called by von Szent-Györgyi hexuronic acid) by the enzyme.

Tauber and Kleiner (136) isolated a powerful enzyme from the pericarp of the Hubbard squash (*Cucurbita maxima*) which is quite different from the one described by von Szent-Györgyi. It oxidizes ascorbic acid very rapidly and completely without the intermediary action of another substance. Oxygen is required for the reaction, since in an atmosphere of N_2 no oxidation of the ascorbic acid by the enzyme takes place. After oxidation of the ascorbic acid by the oxidase the former may be reduced by H_2S, and when the H_2S is removed by N_2 its original reducing power is regained. This enzyme is sensitive to KCN but not to CO and H_2S. The purified enzyme (137) gives no color reaction with benzidine, guaiacol, pyrogallol, catechol, phloroglucinol, and related compounds, with or without H_2O_2. It does not oxidize glutathione, cysteine, tyrosine, adrenalin, or glucose boiled with alkali. Solutions of the enzyme keep well if preserved with toluene and stored in the refrigerator.

This oxidase could not be found in extracts of various mammals' tissues and in cow's milk, or in yeast extracts (137*a*).

The ascorbic acid oxidase oxidizes ascorbic acid probably by introducing two OH groups at the double bond (see formulas). The liberated hydrogen combines with atmospheric oxygen.



Reduced form of ascorbic acid

Oxidized form of ascorbic acid

Optimum *p*H. The optimum is between 5.56 and 5.93 with 0.15 *M* phosphate-citrate buffer and between 5.38 and 5.57 with Na acetate buffer (137). The enzyme is only slightly active below *p*H 4.0 and above *p*H 7.0, thus showing a limited range of activity restricted to the acid side of the *p*H scale. It is less resistant to H ions than to OH ions. The kinetics of ascorbic acid oxidase shows that the action is that of a single enzyme.

Determination. The oxidation may be followed by titration with the oxidation-reduction indicator, sodium 2,6-dichlorobenzenone indophenol. An approximately 0.1 per cent aqueous solution of the dye is standardized against pure ascorbic acid. It is best to extract 0.1 gram of the indicator with several portions of boiling H_2O, making a total of 100 cc. This solution, however, keeps only four days. The ascorbic acid itself oxidizes to some degree in a few hours. A method for the preparation of a more stable standard has been described by Tauber and Kleiner (138). In the same paper a colorimetric method for the estimation of ascorbic acid is given. Iodometric titration may also be employed.

The Interaction of Glutathione in the Enzymic Oxidation of Ascorbic Acid. The experiments of Hopkins and

Morgan (138*a*) have shown that when ascorbic acid and the reduced form of glutathione are together under the influence of ascorbic acid oxidase, the ascorbic acid is completely protected from oxidation. Hydrogen is transferred from two molecules of glutathione to each oxidized molecule of ascorbic acid, keeping it thus in its reduced state. Only when all the glutathione is oxidized does the oxidation of ascorbic acid commence. Glutathione alone is not attacked by the oxidase (137, 138*a*).

Other Vitamins Affecting Enzymes

Ascorbic Acid Dehydrogenase

Roe and Barnum (138*b*) have recently found in human and rat blood plasma and blood cells an enzyme which reduces the reversibly oxidized form of ascorbic acid, thus acting in just the opposite manner from the ascorbic acid oxidase of plants. Table XVIII represents the effect of the reducing enzyme on reversibly oxidized ascorbic acid. Experiments 4, 5, and 6 show that NaF has a very toxic effect on the enzyme. Chloroform and ether, however, are only slightly toxic (experiments 10 and 12). This enzyme apparently plays an important rôle in biological oxidations and reductions.

A Vitamin-A-" Destroying " Enzyme

It is generally known that the drying of alfalfa by means of mechanical driers preserves the vitamin A potency, whereas field curing is destructive to the vitamin. Hauge (139) has shown that alfalfa contains an enzyme which has a destructive action upon the vitamin A factor of this plant and that the sun's rays were not directly destructive during field curing. Figure 25 shows the relation of enzyme action to the destruction of the vitamin A value of alfalfa. Sample 1 has been treated in an autoclave in the presence of live steam, at 17 lb. pressure, for one hour in order to destroy the enzymes, and then dried in the direct sun. In sample 2 the enzyme was inactivated as in sample 1 and then the alfalfa was incubated at 37° for twenty-four hours, and dried in the sun. In sample 3, the enzyme was destroyed by autoclaving the alfalfa; after cooling, potato juice was added, and kept at 37° for twenty-four hours, and dried. This experiment shows that potatoes contain a vitamin-A-decomposing enzyme. Sample 4 was frozen at −25° so as to rupture the plant cells and liberate the enzyme. The alfalfa was then defrosted and kept at 4° for twenty-four hours, after which it was autoclaved and dried. Sample 5 was treated similarly to sample 4, except that it was kept at 37° for twenty-four hours. Sample 6 shows enzyme activity at 4°, sample 7 at 25°, and sample 8 at 37° respectively for a twenty-four-hour incubation period. All samples were dried and finely ground in a mill for the biological assays. The vitamin A units are those of Sherman and Munsell (1925).

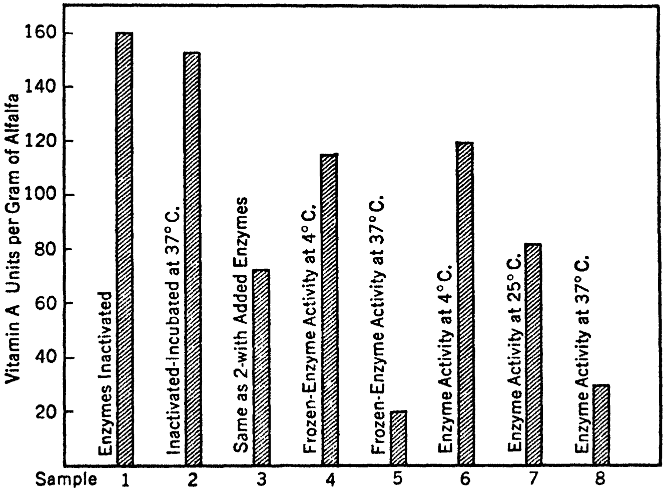


FIG. 25.—Showing the relation of enzyme activity to the destruction of the vitamin A value of alfalfa

A study of the chemical nature of this reaction would be an important contribution to enzymology. It would be interesting to see whether this reaction is reversible and whether the enzyme is specific. For such studies, however, a purified enzyme preparation should be employed.

TABLE XVIII

Effect of Blood on Reversibly Oxidized Ascorbic Acid. Indophenol Titration of Oxalated Blood after 3 Hours' Incubation at 38° C. with 1 Mg. of Reversibly Oxidized Ascorbic Acid Per Cc. The Titrations Were Made upon 5 Cc. of Fluid, except in Experiment 9

Experiment	2,6-Dichlorophenolindophenol titration		
	Total	Blank	Amount due to reduced form of the vitamin
	cc.	cc.	cc.
1. Human plasma	13.4	2.8	10.6
2. Human serum	8.8	1.8	7.0
3. Centrifugate from human erythrocytes washed ten times with 0.9 per cent NaCl solution.	10.1	2.8	7.3
4. Human plasma plus 1 mg. NaF per cubic centimeter	10.9	2.9	8.0
5. Human plasma plus 2 mg. NaF per cubic centimeter	2.9	2.9	0
6. Human plasma plus 5 mg. NaF per cubic centimeter	2.9	2.9	0
7. Human plasma, control on 4, 5, and 6	13.0	2.9	10.1
8. Human plasma, heated 5 minutes at 100° C.	3.4	3.4	0
9. 50 cc. tungstic acid filtrate, 1 : 10 dil.	1.5	1.0	0.5
10. Human plasma plus 1 cc. $CHCl_3$	14.5	3.4	11.1
11. Human plasma control on 10	16.8	3.4	13.4
12. Human plasma plus 1 cc. ethyl ether	11.3	1.8	9.5
13. Human plasma control on 12	11.2	1.8	9.4
14. Guinea-pig plasma	14.0	2.5	11.5
15. Rat blood, erythrocyte centrifugate, 0.9 per cent NaCl	9.0	2.5	6.5

Some of the Functions of Cytochrome in Relation to Oxidases

Cytochrome is an intracellular pigment present in aerobic bacteria, yeast, plants, and animals. It was first noticed by MacMunn (140, 141), who named it histohemin. Hoppe-Seyler (142) contradicted the findings of MacMunn. As the result of recent work of Keilin (130), however, the properties and chemistry of this pigment are now well known. He named it cytochrome and found that it exists in an oxidized and reduced form, and that the reduced form shows characteristic absorption spectra. Keilin showed that cytochrome is a mixture of three independent hemochromogen-like substances, *a*, *b*, and *c*, each with individual oxidizing and reducing properties. Oxygen activated by indophenol oxidase oxidizes cytochrome. The dehydrogenase system of the cell in turn reduces it (143). Thus cytochrome seems to have an intermediary function between the oxygen activators and hydrogen activators of the cell.

Some very interesting experiments which explain at least one function of cytochrome have been published by Harrison (144). In a system of glucose dehydrogenase plus coenzyme and cytochrome *c* plus cytochrome (indophenol) oxidase (of heart muscle), aerobic oxidation of glucose occurs. Otherwise oxidation is negligible. The O_2 uptake in 170 minutes at 37° is given in the following:

1.	Dehydrogenase and coenzyme+glucose	19	cmm.O_2
2.	“ “ “ + “ +oxidase	0	“ “
3.	“ “ “ + “ +cytochrome	7	“ “
4.	“ “ “ + “ + “ oxidase	101	“ “
5.	Glucose+cytochrome+oxidase	18	“ “

Harrison believes that this is probably the mechanism of aerobic oxidation of glucose by glucose dehydrogenase in the body.

Keilin (145) found that cysteine oxidation *in vitro* is greatly accelerated by the indophenol oxidase in the presence of cytochrome.

The kinetics of oxidizing enzymes has recently been discussed by von Euler (146).

REFERENCES

1. Wieland, H.: Über den Verlauf der Oxydationsvorgänge. *Ber.*, **55**, 3639 (1922). Über den Mechanismus der Oxydationsvorgänge. *Ergebnisse d. Physiol.*, **20**, 477 (1922).
2. Warburg, O.: Über Eisen, den Sauerstoff-übertragenden Bestandteil des Atmungsferments. *Ber.*, **58**, 1001 (1925).
3. Warburg, O.: Iron, the oxygen-carrier of respiration-ferment. *Science*, **61**, 575 (1925).
4. Thunberg, T.: Die colorimetrische Vakuum-Mikromethode für das Studium der Wasserstoff aktivierenden Stoffwechsel Enzyme in Oppenheimer-Pincussen; Die Fermente und ihre Wirkungen, **3**, 1118 (1929).
5. Harrison, D. C.: The dehydrogenases of animal tissues. *Ergebnisse Enzymforschung*, **4**, 297 (1935).
6. Ohlsson, E.: Die Abhängigkeit der Wirkung der Succinodehydrogenase von der Wasserstoffionenkonzentration. *Skand. Arch. Physiol.*, **41**, 77 (1921).
7. Thunberg, T.: Zur Kenntnis der Spezifität der Dehydrogenasen. *Biochem. Z.*, **258**, 48 (1933).
8. Einbeck, H.: Über das Vorkommen der Fumarsäure im frischen Fleische. *Z. physiol. Chem.*, **90**, 301 (1941).
9. Fleisch, A.: Some oxidation processes of normal and cancer tissue. *Biochem. J.*, **18**, 294 (1924).

9*a*. Szent-Györgyi, A. von.: Über den Mechanismus der Succin- und Paraphenylendiaminoxydation. Ein Beitrag zur Theorie der Zellatmung. *Biochem. Z.*, **150**, 195 (1924).

10. Stern, L.: Àpropos du mécanisme d'action des catalyseurs oxydants. *Comp. rend. soc. biol.*, **98**, 1288 (1928).
11. Hahn, A., and Harmann, W.: Über die Dehydrierung der Bernsteinsäure. *Z. Biol.*, **89**, 159 (1929).
12. Dixon, M.: The action of carbon monoxide on certain oxidizing enzymes. *Biochem. J.*, **21**, 1211 (1927).
13. Keilin, D.: Cytochrome and respiratory enzymes. *Proc. Roy. Soc. London*, **104**, Series B, 206 (1928–29).
14. Euler, H. von, Myrbäck, K., and Nilsson, R.: Die Co-Zymase. *Ergebnisse Physiol.*, **26**, 553 (1928).

15. Quastel, J. H., and Whetham, M. D.: The equilibria existing between succinic, fumaric, and malic acids in the presence of resting bacteria. *Biochem. J.*, **18**, 519 (1924).
16. Thunberg, T.: Das Reduktions-Oxydationspotential eines Gemisches von Succinat-Fumarat. *Skand. Arch. Physiol.*, **46**, 339 (1924–25).
17. Sen, K. C.: The effect of narcotics on some dehydrogenases. *Biochem. J.*, **25**, 849 (1931).
18. Holmberg, C. G.: Zur Kenntnis des Einflusses von Adenylsäuren auf Gewisse enzymatische, speziell oxydative Prozesse in Muskelextrakt. *Skand. Arch. Physiol.*, **68**, 1 (1934).
19. Hahn, A.: Über Dehydrierungsvorgänge in Muskel. *Z. Biol.*, **92**, 355 (1931–32).
20. Meyerhof, O.: Über die Atmung der Froschmuskulatur. *Pflügers Arch.*, **175**, 20 (1919).
21. Szent-Györgyi, A. von: Zellatmung (Zweite Mitteilung. Der Oxydationsmechanismus der Milchsäure). *Biochem. Z.*, **157**, 50 (1925).
22. Banga, I., Szent-Györgyi, A. von, and Vargha, L.: Über das Co-Ferment der Milchsäureoxydation. *Z. physiol. Chem.*, **210**, 228 (1932).
23. Boyland, E.: Studies in tissue metabolism. I. Vitamin B, and the Coenzyme of Lactic Dehydrogenase. *Biochem. J.*, **27**, 786 (1933).
24. Holmberg, C. G.: Zur Kenntnis des Einflusses von Adenylsäuren auf gewisse enzymatisch, speziell oxydative Prozesse in Muskelextrakt. *Skand. Arch. Physiol.*, **68**, 63 (1934).
25. Boyland, E., and Boyland, M. E.: Studies in tissue metabolism. V. The lactic dehydrogenases of yeast and heart-muscle. *Biochem. J.*, **28**, 1417 (1934).
26. Birch, T. W., and Mann, P. V. G.: The activation of lactic dehydrogenase and its relation to the role of vitamin B. *Biochem. J.*, **28**, 622 (1934).
27. Gözsy, B., and Szent-Györgyi, A. von: Über den Mechanismus der Hauptatmung des Taubenbrustmuskels. Allgemeines über das Koferment und Succinat. *Z. physiol. Chem.*, **224**, 3 (1934).
28. Szent-Györgyi, A. von: The action of arsenite on tissue respiration. *Biochem. J.*, **24**, 1723 (1930).
29. Banga, I., Schneider, L., and Szent-Györgyi, A. von: Über den Einfluss der arsenigen Säure auf die Gewebsatmung. *Biochem. Z.*, **240**, 462 (1931).
30. Banga, I., and Szent-Györgyi, A. von: Über Co-Fermente Wasserstoffdonatoren und Arsenvergiftung der Zellatmung. *Biochem. Z.*, **246**, 203 (1932).

31. Banga, I., and Szent-Györgyi, A. von: Über das Co-Ferment der Milchsäureoxydation. *Z. Physiol.*, **217**, 39 (1933).

32. Andersen, B.: Die Co-Zymase als Co-Enzym bei enzymatischen Dehydrierungen. *Z. physiol. Chem.*, **225**, 57 (1934).

33. Wishart, G. M.: On the reduction of methylene blue by tissue extracts. *Biochem. J.*, **17**, 103 (1933).

34. Harrison and Thurlow: quoted by Harrison (5).

35. Banga, I., Laki, K., and Szent-Györgyi, A. von: Über die Oxydation der Milchsäure und der β-Oxybuttersäure durch den Herzmuskel. *Z. physiol. Chem.*, **217**, 43 (1933).

36. Thunberg, T.: Studien über die Beeinflussung des Gasaustausches des überlebenden Froschmuskels durch verschiedene Stoffe. *Skand. Arch. Physiol.*, **24**, 73 (1910).

37. Butterworth, J., and Walker, T. K.: A study of the mechanism of the regradation of citric acid by *B. pyocyaneus*. *Biochem. J.*, **23**, 926 (1929).

38. Wieland, H., and Sonderhoff, R.: Über den Mechanismus der Oxydationsvorgänge. XXXIV. Die anaerobe Vergärung der Citronensäure durch Hefe, *Ann.*, **503**, 61 (1933).

39. Bernheim, F.: The specificity of the dehydrases. The separation of the citric acid dehydrase from liver and of the lactic acid dehydrase from yeast. *Biochem. J.*, **22**, 1178 (1928).

40. Harrison, D. C.: The oxidation of hexose-diphosphoric acid by an enzyme from animal tissues. *Biochem. J.*, **25**, 1011 (1931).

41. Andersen, B.: Über Co-Zymaseaktivierung einiger Dehydrogenasen. *Z. physiol. Chem.*, **217**, 186 (1933).

42. Battelli, F., and Stern, L.: L'alcoolase dans les tissus animaux. *Comp. rend. soc. biol.*, **67**, 419 (1909).

43. Wieland, H., and Frage, K.: Zur Kenntnis der Leber Dehydrase (Zweiundzwanzigste Mitteilung. Über den Mechanismus der Oxydationsvorgänge). *Z. physiol. Chem.*, **186**, 195 (1928–29).

44. Mizusawa, H.: Beiträge zur Kenntnis der Alkoholoxydase. *J. Biochem.* (Tokyo), **18**, 243 (1933).

45. Lehmann, J.: Aktivierung von Alkoholdehydrogenase in Muskel,- Leber- und Tumorgeweben durch Coenzym. *Biochem. Z.*, **272**, 144 (1934).

46. Müller, D.: Alkoholdehydrase aus Hefe II. *Biochem. Z.*, **268**, 152 (1934).

46*a*. Euler, H. von, and Adler, E.: Über die Komponenten der Dehydrasesysteme. I. Zur Kenntnis der Dehydrierung von Alkohol und Robison-ester. *Z. physiol. Chem.*, **226**, 195 (1934).

47. Lehmann, J.: Aktivierung von Hefealkoholdehydrogenase durch Co-Enzym. *Biochem. Z.*, **272**, 95 (1934).

48. Davies, D. R., and Quastel, J. H.: Dehydrogenations by brain tissue. The effects of narcotics. *Biochem. J.*, **26**, 1672 (1932).
49. Broman, T.: Die Verwendung von Succinodehydrogenase in Muskelextrakt zum Nachweis von Bernsteinsäure. *Skand. Arch. Physiol.*, **59**, 25 (1930).
50. Thunberg, T.: Citric acid in animal fluids. *Am. J. Physiol.*, **90**, 540 (1929).
51. Euler, H. von, and Nilsson, R.: Über die biologische Oxydo-Reduktion. *Skand. Arch. Physiol.*, **59**, 201 (1930).
52. Schardinger, F.: Über das Verhalten der Kuhmilch gegen Methylenblau und seine Verwändung zur Unterscheidung von ungekochter und gekochter Milch. *Z. Unters. Nahr. Genuss.*, **5**, 113 (1902); *Chem. Ztg.*, **28**, 1113 (1908).
53. Morgan, E. J., Stewart, C. P., and Hopkins, F. G.: On the anaerobic and Aerobic oxidation of xanthin and hypoxanthin by tissues and by milk. *Proc. Roy. Soc. London (B)*, **94**, 109 (1922–23).
54. Morgan, E. J.: The distribution of xanthine oxidase. I. *Biochem. J.*, **20**, 1282 (1926).
55. Sen, K. C.: The effect of narcotics on some dehydrogenases. *Biochem. J.*, **25**, 849 (1931).
56. Wieland, H., and Mitchell, W.: Über den Mechanismus der Oxydationsvorgänge. XXIX. Über die Dehydrierenden Enzyme der Milch. IV. *Ann.*, **492**, 156 (1931–32).
57. Clift, F. P., and Cook, R. P.: Triose dehydrogenase. I. *Biochem. J.*, **26**, 1804 (1932).
58. Harrison, D. C.: Glucose dehydrogenase: A new oxidizing enzyme from animal tissues. *Biochem. J.*, **25**, 1016 (1931).
59. Mann, P. J. G.: The reduction of glutathione by a liver system. *Biochem. J.*, **26**, 785 (1932).
60. Bernhauer, K.: Biochemie der oxydativen Gärungen. *Ergebnisse Enzymforschung*, **3**, 185 (1934).
61. Harrison, D. C.: The dehydrogenases of animal tissues. *Ergebnisse Enzymforschung*, **4**, 297 (1935).
62. Lundsgaard, E.: Weitere Untersuchungen über die Einwirkung der Halogenessigsäuren auf den Spaltung- und Oxydationsstoffwechsel. *Biochem. Z.*, **250**, 61 (1932).
63. Meyerhof, O.: Intermediate products and the last stages of carbohydrate breakdown in the metabolism of muscle and in alcoholic fermentation. *Nature*, **132**, 337, 373 (1933).
64. Harrison, D. C.: The chemical nature of the active group in the enzyme glucose dehydrogenase. *Proc. Roy. Soc. London (B)*, **113**, 150 (1933).
65. Harrison, D. C.: Glucose dehydrogenase: Preparation and some

properties of the enzyme and its coenzyme. *Biochem. J.*, **27**, 382 (1933).

66. Andersen, B.: Die Co-Zymase als Co-Enzym bei enzymatischen Dehydrierungen. *Z. physiol. Chem.*, **225**, 57 (1934).
67. Wishart, G. M.: On the reduction of methylene blue by tissue extracts. *Biochem. J.*, **17**, 103 (1923).
68. Harrison, D. C.: Thesis, Cambridge, 1925, p. 27.
69. Needham, D.: Quantitative study of succinic acid in muscle; glutamic and aspartic acids as precursors. *Biochem. J.*, **24**, 208 (1930).
70. Krebs, H. A.: Weitere Untersuchungen über den Abbau der Aminosäuren im Tierkörper. *Z. physiol. Chem.*, **218**, 157 (1933).
71. Bernheim, F., and Bernheim, M. L. C.: The oxidation of proline and oxyproline by liver. *J. Biol. Chem.*, **96**, 325 (1932).
72. Bernheim, F., and Bernheim, M. L. C.: The oxidation of proline and alanine by certain tissues. *J. Biol. Chem.*, **106**, 79 (1934).

72*a*. Ahlgren: *Skand. Arch. Physiol.*, Supplement to Vol. **47** (1925).

72*b*. Green, D. E., and Dixon, M.: Studies on xanthine oxidase. XI. Xanthine oxidase and lactoflavine. *Biochem. J.*, **28**, 237 (1934).

72*c*. Dixon, M.: Manometric Methods as Applied to the Measurement of Cell Respiration and Other Processes. Macmillan, New York. 1934.

73. Hand, D. B.: Peroxidase. A comparison with other iron-porphyrin catalysts in cells. *Ergebnisse Enzymforschung*, **2**, 272 (1933).

73*a*. Bancroft, G., and Elliott, K. A. C.: The distribution of peroxidase in animal tissues. *Biochem. J.*, **28**, 1911 (1934).

74. Dakin, H. D.: Oxidation and Reduction in the Animal Body. Longmans, Green and Co., London, 1922.
75. Keilin, D.: Cytochrome and intracellular oxidase. *Proc. Roy. Soc. London (B)*, **106**, 418 (1930).
76. Thurlow, S.: Studies on xanthine oxidase. *Biochem. J.*, **19**, 175 (1925).
77. Kodama, K.: Studies on xanthine oxidase. *Biochem. J.*, **20**, 1095 (1926).
78. Onslow, M. W., and Robison, M. E.: Oxidising enzymes. On the mechanism of plant oxidases. *Biochem. J.*, **20**, 1138 (1926).
79. Hare, M. L. C.: Tyramine oxidase. A new enzyme system in the liver. *Biochem. J.*, **22**, 968 (1928).
80. McLeod, J., and Gordow, J.: Production of hydrogen peroxide by bacteria. *Biochem. J.*, **16**, 499 (1922).
81. Avery, O. T., and Neil, J. M.: The antigenic properties of solutions of pneumococcus, *J. Exptl. Med.*, **42**, 355 (1925).
82. Wieland, H., and Rosenfeld, B.: Über den Mechanismus der Oxidationsvorgänge. *Ann.*, **477**, 32 (1930).
83. Elliott, K. A. C. Milk peroxidase. *Biochem. J.*, **26**, 10 (1932).

84. Balls, A. K., and Hale, W. S.: On peroxidase. *J. Biol. Chem.*, **107**, 767 (1934).

85. Bach, A., and Chodat, R.: Untersuchungen über die Role der Peroxidase. *Ber.*, **37**, 1342 (1904).

86. Wolff, J., and Stoecklin, E. D.: L'oxyhemoglobine peut-elle fonctionner comme peroxydase. *Ann. Inst. Pasteur*, **25**, 313 (1911).

87. Willstätter, R., and Pollinger, A.: Über die peroxydatische Wirkung der Oxyhämoglobine. *Z. physiol. Chem.*, **130**, 281 (1923).

88. Kuhn, R., and Brann, L.: Über die Abhängigkeit der katalitischen und peroxydatischen Wirkung des Eisens von seiner Bindungsweise. *Ber.*, **59**, 2370 (1926).

89. Alsberg, C. L.: Beiträge zur Kenntnis der Guajak-Reaktion. *Arch. exptl. Path. Pharmakol.*, *Supplement-Band*, **39** (1908).

90. Batteli, F., and Stern, L.: Oxydation des *p*-phenylendiamins durch die Tiergewebe. *Biochem. Z.*, **46**, 317, 342 (1912).

91. Pugh, C. E. M., and Raper, H. S.: The action of tyrosinase on phenols. With some observations on the classification of oxidases. *Biochem. J.*, **21**, 1370 (1927).

92. Willstätter, R., and Heiss, H.: Constitution of purpurogallin. *Ann.*, **183**, 17 (1923).

92*a*. Willstätter, R.: Über Sauerstoff-Übertragung in der lebenden Zelle. *Ber.*, **59**, 1871 (1926).

93. Tauber, H.: The interaction of peroxidase and ascorbic acid (vitamin C) in biological oxidations and reductions. *Enzymologia*, **1**, No. 4 (1936).

93*a*. Borsook, H., and Jeffreys, C. E. P.: Glutathione and ascorbic acid. *Science*, **83**, 397 (1936).

94. Willstätter, R., and Pollinger, A.: Bemerkungen über Peroxidase aus Getreidekeimen. *Untersuchungen über Enzyme*, 522 (1928).

95. Willstätter, R., and Stoll, A.: Peroxydasen. *Ann.*, **416**, 21 (1917–18).

96. Willstätter, R., and Weber, H.: Über Hemmung der Peroxydase durch Hydroperoxyd. *Ann.*, **449**, 175 (1926).

96*a*. Willstätter, R., and Weber, H.: Zur quantitativen Bestimmung der Peroxydase. *Ann.*, **449**, 175 (1926).

97. Ucko, H., and Banshi, H. W.: Über Peroxydase (Dritte Mitteilung. Zur Kinetik der Peroxydase). *Z. physiol. Chem.*, **159**, 235 (1926).

98. Kuhn, R., Hand, D. B., and Florkin, M.: Über die Natur der Peroxydase. *Z. physiol. Chem.*, **201**, 255 (1931).

99. Elliott, K. A. C., and Sutter, H.: Die Einwirkung von Kohlenoxyd auf Peroxydase. *Z. physiol. Chem.*, **205**, 47 (1932).

99a. Sumner, J. B., and Howell, S. F.: Hematin and the peroxidase of fig sap. *Enzymologia*, **1**, 133 (1936).

100. Bourquelot, E., and Bertrand, G.: Le bleuissement et le noircissement des champignons. *Compt. rend. soc. biol.*, **47**, 582 (1895).

101. Bertrand, G.: Chimie physiologique—Sur une nouvelle oxydase, ou ferment soluble oxydant, d'origine végétale. *Compt. rend. acad. sci.*, **122**, 1215 (1896).

102. Bertrand, G.: Chimie physiologique—Action de la tyrosinase sur quelques corps voisins de la tyrosine. *Compt. rend. acad. sci.*, **145**, 1352 (1907).

103. Chodat, R.: Oxidizing enzymes. *Ann. sci. phys. nat.*, **33**, 70 (1912).

104. Chodat, R., and Staub, M.: Oxidizing ferments. I. The mood of action of tyrosinase. *Arch. phys. nat.*, **23**, 265 (1907).

105. Happold, F. C., and Raper, H. S.: The tyrosinase-tyrosine reaction. III. The supposed deaminising action of tyrosinase on amino acids. *Biochem. J.*, **19**, 92 (1925).

106. Robinson, M. B., and McCance, R. A.: Oxidative deamination by a basidiomycete enzyme. *Biochem. J.*, **19**, 251 (1925).

107. Szent-Györgyi, A. von: Zellatmung (Vierte Mitteilung. Über den Oxydationsmechanismus der Kartoffeln). *Biochem. Z.*, **162**, 399 (1925).

108. Pugh, C. E. M., and Raper, H. S.: The action of tyrosinase on phenols. With some observations on the classification of oxidases. *Biochem. J.*, **21**, 1370 (1927)

109. Raper, H. S.: Tyrosinase. *Ergebnisse Enzymforschung*, **1**, 270 (1932).

110. Bloch, B.: Chemische Untersuchungen über das spezifische pigmentbildende Ferment der Haut, die Dopaoxydase. *Z. physiol. Chem.*, **98**, 226 (1917).

111. Bloch, B., and Schaaf, F.: Pigmentstudien. *Biochem. Z.*, **162**, 181 (1925).

112. Albl, H.: Über das Auftreten von Brenzkatechinderivaten als Pigmentvorstufen (Melanogene) in Harn bei allgemeiner Melanose und den Nachweis des pigmentbildenden Fermentes (Dopaoxydase) im Haut-Pressaft von Kaninchen. Dissertation, Zürich, 1926.

113. Bloch, B., and Schaff, F.: Über die Pigmentbildung in der Haut, unter besonderer Berücksichtigung der optischen Spezifität der Dopaoxydase. *Klin. Wochenschr.*, **11**, 10 (1932).

114. Peck, S. M., Sobotka, H., and Kahn, J.: Zur optischen Spezifität der Dopaoxydase. *Klin. Wochenschr.*, **11**, 14 (1932).

115. Mulzer, P., and Schmalfuss, H.: Das Dunkeln der Haut. 1. Dunklungsvorstufen. 2. Die Bedingungen. *Med. Klin.*, **27**, 1099 (1931); **29**, 732 (1933).

116. Schmalfuss, H., and Schmalfuss, Z.: Über das Absgestimmtsein von Anregern, am Beispiel des Dunkelns. *Biochem. Z.*, **263**, 278 (1933).

117. Happold, F. C.: The correlation of the oxidation of certain phenols and of dimethyl-*p*-phenylenediamine by bacterial suspensions. *Biochem. J.*, **24**, 1737 (1930).

118. Bertrand, G.: (*a*) Sur les rapports qui existent entre la constitution chimique des composés organiques et leur oxydabilité sous l'influence de la laccase. (*b*) Sur la présence simultanée de la laccase et de la tyrosinase dans le suc de quelques champignons. (*c*) Sur l'oxydation au gayacoi par la laccase. *Comp. rend. acad. sci.*, **122**, 1132 (1896); **123**, 463 (1896); **137**, 1269 (1903).

119. Bergemann, O. H. K.: Beiträge zur Kenntnis pflanzlicher Oxydationsfermente. *Pflügers Arch.*, **161**, 45 (1915).

120. Onslow-Wheldale, M.: (*a*) Oxidising enzymes. IV. The distribution of oxidising enzymes among the higher plants. *Biochem. J.*, **15**, 107. (*b*) Oxidising enzymes. V. Further observations on the oxidising enzymes of fruits. *Biochem. J.*, **15**, 113 (1921).

121. Neuberg, C.: Enzymatische Umwandlung von Adrenalin. *Biochem. Z.*, **8**, 383 (1908).

122. Portier, P.: L'oxydase du sang des mammifères, sa localisation dans le leucocyte. *Compt. rend. soc. biol.*, **50**, 452 (1898).

123. Koga, T.: Über die Fermente im Hühnerei. *Biochem. Z.*, **141**, 430 (1923).

124. Fleury, P.: Rapport entre l'activité diastasique et la réaction du milieu. II. Application a l'étude de la laccase. *Bull. soc. chim. Biol.*, **6**, 560 (1924).

125. Suminskura, K.: Über die Laccase des jananischen Lachs. *Biochem. Z.*, **224**, 292 (1930).

126. Ehrlich, P.: Das Sauerstoff-Bedürfnis des Organismus. Berlin, 1885.

127. Pohl, J.: Zur Kenntniss des oxydativen Fermentes. *Arch. exptl. Path. Pharm.*, **38**, 65 (1896).

128. Batelli, F., and Stern, L.: (*a*) Oxydation des *p*-Phenylendiamins durch die Tiergewebe. (*b*) Einfluss verschiedener Faktoren auf die Oxydation des *p*-Phenylendiamins durch die Tiergewebe. *Biochem. Z.*, **46**, 317, 343 (1912).

129. Keilin, D.: Influence of carbon monoxide and light on indophenol ozidase of yeast cells. *Nature*, **119**, 670 (1927).

130. Keilin, D.: Cytochrome and intracellular respiratory enzymes. *Ergebnisse Enzymforschung*, **2**, 239 (1933).

131. Dixon, M., Hill, R., and Keilin, D.: The absorption spectrum of

the component *c* of cytochrome. *Proc. Roy. Soc. London (B)*, **109**, 29 (1932).

132. Battelli, F., and Stern, L.: Untersuchungen über die Uricase in Tiergeweben. *Biochem. Z.*, **19**, 219 (1909).

133. Carey, P. C., and Smith, J. C.: Higher aliphatic compounds. *J. Chem. Soc.*, 635 (1933).

134. Szent-Györgyi, A. von: On the mechanism of biological oxidation and the function of the suprarenal gland. *Science*, **72**, 125 (1930).

135. Szent-Györgyi, A. von: On the function of hexuronic acid in the respiration of the cabbage leaf. *J. Biol. Chem.*, **90**, 385 (1931).

136. Tauber, H., and Kleiner, I. S.: Isolation of a specific ascorbic acid (vitamin C) oxidase. *Proc. Soc. Exptl. Biol. Med.*, **32**, 577 (1935).

137. Tauber, H., Kleiner, I. S., and Mischkind, D.: Ascorbic acid (vitamin C) oxidase. *J. Biol. Chem.*, **110**, 211 (1935).

137*a*. The author's unpublished results.

138. Tauber, H., and Kleiner, I. S.: A method for the quantitative determination of ascorbic acid (vitamin C). The vitamin C content of various plant and animal tissues. *J. Biol. Chem.*, **108**, 563 (1935).

138*a*. Hopkins, F. G., and Morgan, E. J.: Some relations between ascorbic acid and glutathione. *Biochemical J.*, **30**, 1446 (1936).

138*b*. Roe, J. H., and Barnum, G. L.: The anti-scorbutic potency of reversibly oxidized ascorbic acid and the observation of an enzyme in blood which reduces the reversibly oxidized vitamin. *J. Nutrition*, **11**, 359 (1936).

139. Hauge, S. M.: Evidence of enzymatic destruction of the vitamin A value of alfalfa during the curing process. *J. Biol. Chem.*, **108**, 331 (1935).

140. MacMunn, C. A.: Further observations on myohaematin and the histohaematins. *J. Physiol.*, **8**, 57 (1887).

141. MacMunn, C. A.: Über das Myohaematin. *Z. physiol. Chem.*, **14**, 328 (1890).

142. Hoppe-Seyler, F.: Über Muskelfarbstoffe. *Z. physiol. Chem.*, **14**, 106 (1890).

143. Keilin, D.: Cytochrome and respiratory enzymes. *Proc. Roy. Soc. London (B)*, **104**, 206 (1929).

144. Harrison, D. C.: Glucose dehydrogenase: A new oxidising enzyme from animal tissues. *Biochem. J.*, **25**, 1016 (1931).

145. Keilin, D.: Cytochrome and intracellular oxidase. *Proc. Roy. Soc. London (B)*, **106**, 418 (1930).

146. Euler, H. von: Die Katalasen und die Enzyme der Oxydation und Reduktion. J. Springer, Berlin, 1934.

CHAPTER VIII

THE FLAVIN OXIDATION SYSTEM OF WARBURG AND CHRISTIAN AND ITS RELATION TO OTHER DYES

Preparation of the Components and Their Properties

In 1932 Von Szent-Györgyi and associates (1, 2) obtained from heart muscle a yellow water-soluble dye. They named it *cytoflav.* The dye could easily be reduced to the leuco form and reoxidized to the original state. Shortly after this discovery of the Hungarian workers, Warburg and Christian (3, 4) announced the isolation of a "yellow ferment" from bottom yeast (Lebedew juice). The aqueous solution of this "enzyme" has a yellow color which disappears if treated with reducing agents but returns if the solution is shaken with oxygen (auto oxidation). The two dyes have been found to be related in chemical nature (5). Whereas cytoflav is lactoflavin phosphoric acid ester, the yellow enzyme is a combination of this ester, with a specific protein.

Cytoflav was obtained by von Szent-Györgyi and coworkers by the extraction of the heart muscle with boiling 1 per cent trichloroacetic acid and treating the filtered and acidified extract with mercuric nitrate. The precipitate which formed was washed with water, methyl alcohol, acetone, and finally with a mixture of HCl, acetone, and ether. The washed residue was then suspended in water, acidified with HCl, and treated with saturated $HgCl_2$. The filtrate contained the cytoflavin and was concentrated in vacuum and precipitated with acetone. The precipitated pigment they then washed with acetone. It has a golden yellow color. Probably a colorless specific substance (coenzyme) is precipitated together with the dye.

In contrast to the preparation of cytoflavin, the yellow ferment is obtained at low temperature. In it flavin phosphoric acid is bound to a protein substance.

The following are the main steps used by Warburg and Christian for the *preparation of the yellow ferment*: The fresh juice of bottom yeast (Lebedew) was treated with lead subacetate and the excess lead removed by the addition of phosphate. An equal volume of acetone was added and the mixture filtered. The filtrate contained the enzyme. The filtrate was then saturated with CO_2 at 0° C. The pigment enzyme precipitated in the form of a yellow oil. By repeating the precipitation from an aqueous solution with acetone and CO_2, followed by precipitation from aqueous solution by methyl alcohol, the substance was obtained in the form of a yellow powder.

According to Warburg and Christian, *B. delbruckii* contains large amounts of the yellow ferment.

The oxidation of hexose monophosphoric acid by the yellow ferment requires a *coenzyme* which may be prepared from red blood cells and a *second enzyme* which like the yellow ferment may be obtained from Lebedew juice of bottom yeast in the following manner: Lebedew juice is diluted 100 times with water and saturated with CO_2. The precipitate formed is dissolved in a volume of bicarbonate solution equal to the original Lebedew juice. The resulting solution is employed as a second enzyme.

HCN does not affect the oxygen-transferring system of Warburg and Christian. Until quite recently, only one substrate has been employed, the hexosemonophosphate of Robison.

Euler and Adler (6) showed, however, that if, to the oxidation system of Warburg and Christian, adenosintriphosphoric acid (from muscle) is added it acquires the ability to oxidize hexoses. An esterification of hexose to hexosemonophosphate is suggested as an intermediary stage. Adenosintriphosphoric acid cannot be replaced by other substances like creatin, phosphagen, sodium pyrophosphate, or hexosediphosphate. These authors separated the *second enzyme* (Zwischenferment) of the oxidation system into two enzymic components. One is the dehydrase which, in the presence of the flavin enzyme and coenzyme, oxydizes hexosemonophosphate but not fructose. The other enzyme in the presence of adenosintriphosphoric acid oxidizes hexoses. The mixture is a typical dehydrogenase system (Wieland) using as a hydrogen acceptor the "yellow ferment" from bottom yeast.

Crystallization of the Yellow Enzyme

Theorell (7), working in Warburg's laboratory, reported recently on the crystallization of the yellow ferment. He obtained by the method of Warburg and Christian about 30 grams of the crude enzyme which he further purified by cataphoresis at *p*H 4.2 to 4.5. This procedure caused a loss of only 10 per cent. By fractionation with ammonium sulfate at *p*H 5.2 and dialysis against 2 volumes of saturated ammonium sulfate plus 1 volume acetate buffer of *p*H 5.2, a crystalline product was obtained yielding 60 per cent. The constancy in pigment content indicated that the substance is pure. It contained 15.5 per cent N.

Crystalline Dye Component of the Yellow Enzyme

Figure 26 shows the crystalline dye component of the yellow ferment as obtained by Warburg and Christian (3). The dye has been freed from the protein component. The two compounds are inactive. Theorell (7), however, was able to hydrolyze the yellow enzyme reversibly.

Reversible Hydrolysis of the Enzyme

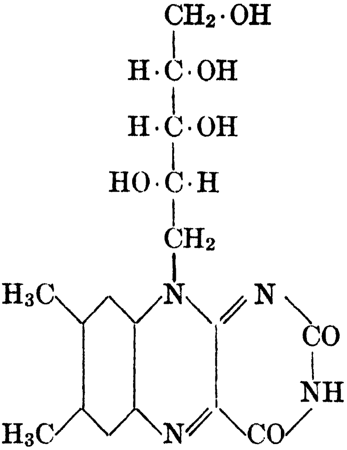
If a salt-free solution of the pure enzyme is dialyzed against diluted HCl, a splitting of the enzyme into dye and protein takes place. The HCl may be separated from the protein by dialysis. The two components by themselves are inactive. Mixing the two results in the reappearance of the yellow color and almost all the activity. It seems that the dye, which is able to combine loosely with the enzyme, acts here as a coenzyme to the protein, which becomes inactive on heating. The dye is heat-stable (7).

FIG. 26.—Crystalline dye component of the yellow enzyme (Macroscopic crystals)

Formation of the Active Chromoprotein from the Synthetic Prosthetic Group and the Specific Protein

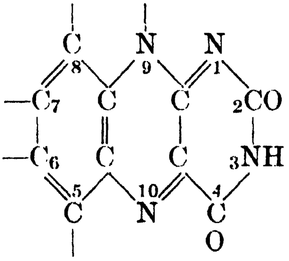
Karrer, Schöpp, and Benz, and Kuhn, Reinemund, Weygand, and Ströbele independently and at the same time reported the synthesis of 6,7-dimethyl-9-(*d*-*l*′-ribityl) isoalloxazine, which is in every respect identical with lactoflavin (vitamin B_2) (discussed in an excellent review by Theorell (8)). It is not certain yet whether lactoflavin is the only antidermatitis or antipellagra factor.

Lactoflavin has the following formula:



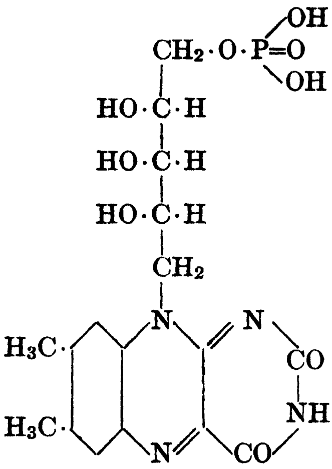
6,7-Dimethyl-9-(*d*-*l*′-ribityl)-isoalloxazine. Vitamin B_2

All flavins contain the flavin nucleus:



Flavin = Isoalloxazine

Vitamin B_2, when combined with phosphoric acid, forms the prosthetic group of the yellow enzyme. Kuhn and Rudy (9) obtained synthetically the prosthetic group, its formula being:



6,7-dimethyl-9-*d*-ribitylisoalloxazin-5′-phosphoric acid

Both the natural flavin phosphoric acid (as isolated from the yellow enzyme) and the synthetic lactoflavin-5′-phosphoric acid are capable of combining with the specific protein of the yellow enzyme, forming chromoproteins of the same catalytic activity (9). These confirm the findings of Warburg and Christian, and those of Theorell. Two different methods were employed by Kuhn and Rudy in their oxidation studies; the Warburg manometric procedure and the Thunberg methylene blue technic.

Borsook (10) calls this type of reaction (the oxidation system) "incomplete enzyme centers." He states, "The non-iron containing respiratory enzyme described by Warburg and Christian, is an example of an incomplete enzyme completed by a yellow pigment, exactly in the manner dehydrogenases are completed by dyes such as methylene blue." In the chromoprotein enzymes the pigment's rôle is that of a coenzyme. The leuco form of the yellow pigment reacts with methylene blue since it has a potential negative to that of methylene blue. Its low potential is of great importance in biological oxidation, since it carries O_2 to metabolites and energy from oxidizing metabolites into a reduction of other metabolites or into a synthesis. This explains the biologic rôle of the pigment vitamin.

Warburg's Apparatus for the Microanalysis of Gases

This apparatus (Fig. 27) is devised to analyze mixtures of gases where the unknown gas constitutes less than 3 per cent of the sample. Any gas may be determined for which an absorbent is available which consists of two components that are inactive until mixed. For instance, estimation of O_2 is made by the use of acidified pyrogallol and KOH, and CO_2 is estimated by the use of $KMnO_4$ and NaI. Warburg's apparatus is very accurate and convenient for oxidase studies (obtainable from American Instrument Company, Silverspring, Md.). For details see the handbook of Dixon (11).

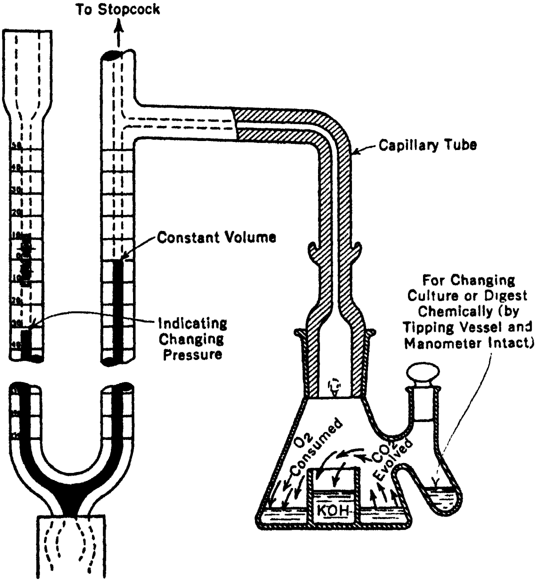

To Stopcock
Capillary Tube
Constant Volume
Indicating Changing Pressure
For Changing Culture or Digest Chemically (by Tipping Vessel and Manometer Intact)
O2 Consumed
CO2 Evolved
KOH

FIG. 27.—Warburg's manometric apparatus

REFERENCES

1. Banga, I., and Szent-Györgyi, A. von: Über Co-Fermente, Wasserstoffdonatoren und Arsenvergiftung der Zellatmung. *Biochem. Z.*, **246**, 203 (1932).
2. Banga, I., Szent-Györgyi, A. von, and Varga, L.: Über das Co-Ferment der Milchsäureoxydation. *Z. physiol. Chem.*, **210**, 228 (1932).
3. Warburg, O., and Christian, W.: Über das neue Oxydationsferment. *Naturwiss.*, **20**, 980 (1932).
4. Warburg, O., and Christian, W.: Über ein neues Oxydationsferment und sein Absorptionsspektrum. *Biochem. Z.*, **254**, 438 (1932).
5. Szent-Györgyi, A. von: Non-enzymic catalysts of cellular oxidation. *Arch. exptl. Zellforschung.*, **15**, 29 (1934).
6. Euler, H. von, and Adler, E.: Über die Komponenten der Dehydrasesysteme. VI. Dehydrierung von Hexosen unter Mitwirkung von Adenosintriphosphorsäure. *Z. physiol. Chem.*, **235**, 122 (1935).
7. Theorell, H.: Reindarstellung (Kristallisation) des gelben Atmungsfermentes und die reversible Spaltung desselben. *Biochem. Z.*, **272**, 155 (1934).
8. Theorell, H.: Das Oxydationsferment. *Biochem. Z.*, **278**, 263 (1935).
9. Kuhn, R., and Rudy, H.: Katalytische Wirkung der Lactoflavin-5′-phosphorsäure; Synthese des gelben Fermentes. *Ber.*, **69**, 1974 (1936).
10. Borsook, H.: Reversible and reversed enzymatic reactions. *Enzymforschung*, **4**, 1 (1935).
11. Dixon, M.: Manometric Methods as Applied to the Measurement of Cell Respiration and Other Processes. Macmillan Co., New York, 1934.

CHAPTER IX

THE ZYMASE COMPLEX AND ALCOHOLIC FERMENTATION

Thirty-five years ago Buchner showed that cell-free yeast juice can also produce alcoholic fermentation, i.e., production of alcohol and CO_2 from sugar. During the past few years a great many studies have been conducted so as to elucidate the differences between cell-free and living yeast. Nothing is known, however, concerning the function of the living cell; nor are certain changes which take place in yeast juice explained as yet. Various yeast preparations (Buchner's yeast juice, Lebedew's extract, Albert's zymin, and dried yeast) ferment sugar more slowly than living yeast cells. When inorganic phosphate is added to these preparations, fermentation is accelerated and phosphoric esters of sugar form (1).

Zymase is a complex of enzymes and coenzymes. The exact number of specific enzymes and coenzymes of the zymase complex is not known. The name zymase represents the effect of a number of enzymes which bring about fermentation. The action of the following enzymes is specific:

1. *Hexokinase* (2) converts fermentable hexoses into a more reactive form (enol?).
2. *Phosphatase* hydrolyzes and synthesizes enol-sugar-phosphoric acid esters. Mg is also an essential activator (3–5).
3. *Oxydoreducase* (mutase, dehydrase) rearranges aldehydes (Cannizzaro reaction) and requires cozymase.
4. *Carboxylase* acts upon pyruvic acid (6). It requires cocarboxylase (7).

The following terminology is used:

Holozymase for the fermentation complex plus all activators. *Apozymase* for cozymase-free holozymase. *Atiozymase* for Mg-free and cocarboxylase-free apozymase (Auhagen).

The zymase complex ferments the simple sugars, *d*-glucose, *d*-fructose, *d*-mannose, *d*-mannononose, glyceric aldehyde, and hydroxyacetone. The last forms fructose (2), diphosphoric acid, and a hexose before it is fermented (8). Galactose is fermented by special yeasts only. Yeast also ferments α-glucosides, phosphoric esters, and disaccharides.

The general belief was that the disaccharides are first hydrolyzed by invertases before they are fermented. Willstätter and Steibelt (9) found that yeast ferments maltose very rapidly, whereas the extract contains hardly any maltase. Willstätter and Oppenheimer (10) fermented lactose with a lactase-free (lactose) yeast. Sucrose was fermented under similar conditions (11). It should be noted, however, that the tests for invertases were carried out on the cell-free extracts.

Estimation of Activity

According to Myrbäck (12), a cell-free fermentation depends upon the following factors:

1. The zymase complex.
2. Some unknown activators of ätiozymase.
3. Inorganic phosphate (Harden).
4. Magnesium (Lohman).
5. Small amounts of hexosediphosphate (13, 14).
6. Cozymase (Harden, Euler-Myrbäck).
7. Optimum *p*H of 6.2 to 6.6 (15).

The volume of CO_2 which forms is estimated in a special apparatus of Myrbäck and Euler (16) or of Warburg. The CO_2 may also be determined gravimetrically.

Willstätter and Steibelt (17) measure the "half-fermentation" time, i.e., the number of minutes necessary for the formation of 50 per cent of the theoretical CO_2.

Induction

When apozymase which contains Mg is mixed with sugar, phosphate, and cozymase, there is sometimes a delay in fermentation. This period of delay is called "induction time." The cause of this induction is not known (18), but can be corrected by the addition of a trace of hexosediphosphoric acid (14, 19).

Cozymase

Harden and Young (20) found that yeast press juice (zymase) becomes more active with the addition of some boiled (enzyme-free) press juice. Active juice loses its potency on dialysis or ultra-filtration but becomes active again with the addition of the filtration residue or the inactive dialysate. Similarly, reactivation could be accomplished by the addition of boiled inactive press juice. Later, the same authors noticed that inorganic phosphate and a certain organic compound of small molecular weight and non-enzymic nature are indispensable for fermentation (21). Von Euler and Myrbäck named the substance cozymase (22). Cozymase-free yeast can readily be obtained by washing yeast with H_2O.

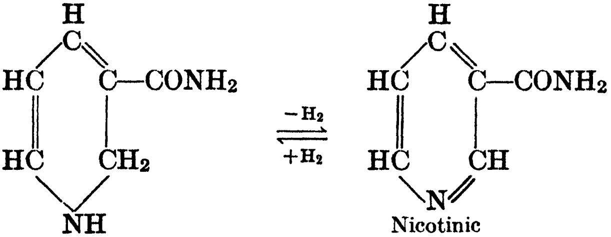
There is some similarity between alcoholic fermentation by yeast and lactic acid formation by muscle juice. Identical esters form. For instance, hexosediphosphoric acid is converted to α-glycerophosphoric acid and phosphoglyceric acid. The addition of fluorides inhibits further decomposition. Phosphates interact. Meyerhof believed that the cozymase of alcoholic fermentation is identical with the muscle cozymase. Lactic acid formation requires the presence of a coenzyme, and this can be replaced by cozymase (23, 24). Recently, however, the identity of yeast cozymase with muscle cozymase has been questioned (25–29).

Cozymase can function as a coenzyme for hexosemonophosphate dehydrogenase and for alcohol dehydrogenase of yeast. Cozymase of yeast and the coenzyme of Warburg and Christian from red cells of the horse are able to replace each other in the hexosemonophosphate dehydrogenase system. The coenzyme of Warburg and Christian cannot replace cozymase in the alcohol dehydrogenase system. It can act, however, as a coenzyme for the liver glucose dehydrogenase, for which cozymase is also a coenzyme (30).

Isolation of Cozymase

Myrbäck (31), in an excellent review on cozymase, describes a method for the preparation and purification of this substance. The purest preparations are colorless and water-soluble. They give negative protein color tests and contain only combined phosphoric acid. Their P content is close to that of adenylic acid, and the pentose test is positive. The N content of the substance is slightly less than that of adenylic acid. On hydrolysis with acids, a nitrogenous base is liberated. This may be obtained in the form of a crystalline picrate which has been identified as adeninepicrate. From this in turn adenine may be prepared. It is probably a mononucleotid, having a molecular weight of 350 (32, 33). Myrbäck (34) showed that there is a reducing group in the cozymase molecule. The activity of the coenzyme parallels the reducing property.

Warburg, Christian, and Griese (35), and Euler, Albers, and Schlenk (35*a*), have shown that *nicotinic acid amide* is the essential component of coenzymes active in fermentation and dehydrogenation. In the course of the enzymic reaction, this pyridine derivative undergoes reversible oxidation-reduction changes involving two hydrogen atoms:



Reduced nicotinic acid amide

Nicotinic acid amide

Although the cozymase of dehydrogenation obtained from red blood cells (35) and the cozymase obtained from yeast (35*a*) are very closely related chemically, they cannot replace each other in their function.

Besides nicotinic acid amide the two coenzymes contain adenine, pentose, and phosphoric acid. The coenzymes are the prosthetic group of the enzymes. They attain their full activity only when combined with a specific protein.

Myrbäck believes that the cozymase becomes a component or part of the enzyme which it activates, just as heme is a part of hemoglobin, and the yellow component is a part of the flavin enzyme.

The Rôle of Inorganic Phosphate in Alcoholic Fermentation

If yeast or muscle juice is added to sugar, two simultaneous reactions take place: fermentation to alcohol (or lactic acid) and carbon dioxide, and conversion into phosphoric esters. Inorganic phosphate plays an important but still unknown rôle in these changes. In the case of yeast, according to Harden and Henly (35*b*, 35*c*), the quantity of sugar fermented is equivalent to the quantity of phosphoric acid esterified in the ratio $C_6H_{12}O_6/2PO_4$, or simply on fermentation one molecule of sugar yields two molecules of carbon dioxide, the carbon dioxide formed being equivalent to the phosphoric acid esterified, $CO_2/PO_4 = 1$. Kluyver and Struyk (36), however, found a ratio of only 0.6. Harden and Young (37) isolated from the fermentation mixture hexosediphosphate of the composition $C_6H_{10}O_4(PO_4H_2)2$. On the formation of this ester they based their views as to what took place in fermentation by yeast' in the presence of phosphate and expressed these in two equations. The first phase is the rapid decomposition during which esterification also takes place; the other is the secondary change when inorganic phosphate is regenerated from hexosediphosphate.

$$(1) \quad 2C_6H_{12}O_6 + 2PO_4HR_2 = 2CO_2 + 2C_2H_6O + C_6H_{10}O_4(PO_4R_2)2 + 2H_2O$$

$$(2) \quad C_6H_{10}O_4(PO_4R_2)2 + 2H_2O = C_6H_{12}O_6 + 2PO_4HR_2$$

Meyerhof (38), however, has shown that hexosediphosphate is directly fermented without glucose formation, so that equation 2 must be corrected:

$$C_6H_{10}O_4(PO_4R_2)2 + 2H_2O = 2CO_2 + 2C_2H_6O + 2PO_4HR_2$$

In 1914 Harden and Robison isolated another hexosephosphoric acid from fermentation mixtures. Robison (39) and Robison and King (40) showed that this ester is hexosemonophosphoric acid. The ratio CO_2/PO_4 esterified is not affected, whether the product consists of mainly one or the other form of the esters. According to Harden, one of the esters is an intermediary product in sugar fermentation. The phosphoric group converts the hexose into a more unstable molecule. He admits, however, that none of the existing theories are satisfactory for the explanation of the intermediate phosphoric esters. This is a difficult task since four different phosphoric esters have been isolated from the fermentation products of hexose in the presence of yeast (38). The importance of phosphate in alcoholic fermentation is beyond doubt.

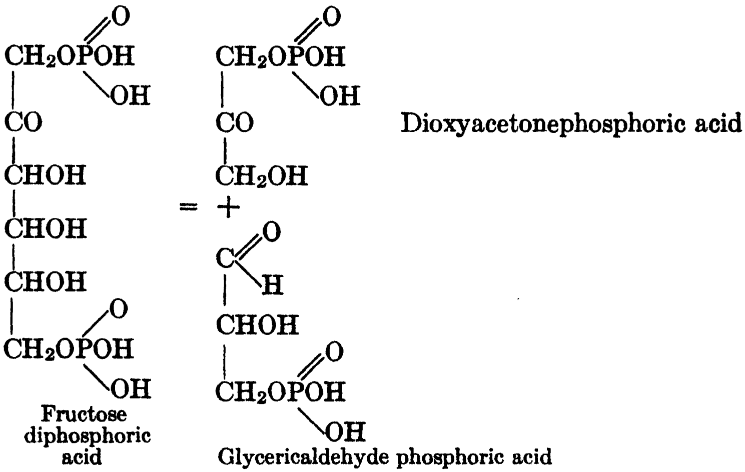
Intermediary Products of Fermentation

Intermediary Products of Lactic Acid Fermentation. Scheme of Embden. In view of their experimental results

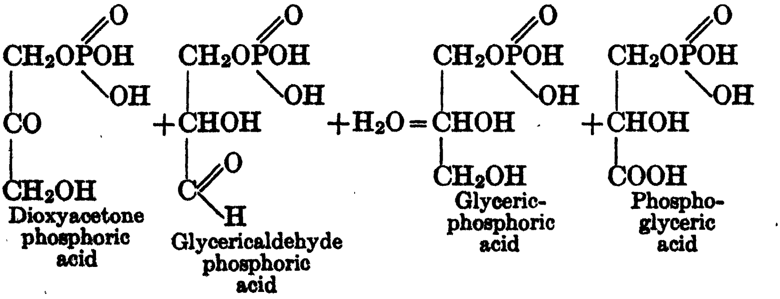
Embden and associates (41) suggested the following scheme for the glucolytic formation of lactic acid by muscle tissue extracts:

Phase I. Synthesis of hexosediphosphoric acid from one molecule of hexose and two molecules of phosphoric acid, or from one molecule of hexosemonophosphoric acid and one molecule of phosphoric acid.

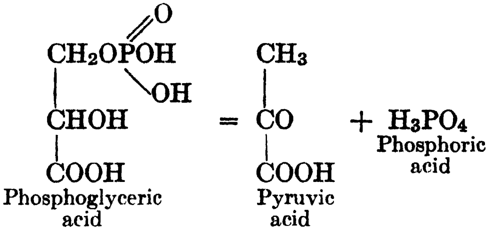
Phase II. Hydrolysis of hexosediphosphoric acid to two molecules of triosephosphoric acid


Fructose diphosphoric acid
Dioxyacetonephosphoric acid
Glycericaldehyde phosphoric acid

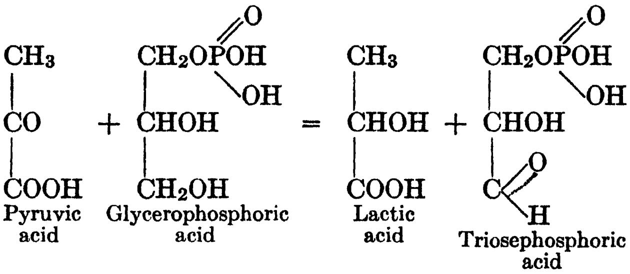
Phase III. By dismutation (Cannizzaro reaction), the two molecules of triosephosphoric acid are converted into one molecule of glycerophosphoric acid and one molecule of phosphoglyceric acid


Dioxyacetone phosphoric acid
Glycericaldehyde phosphoric acid
Glyceric-phosphoric acid
Phospho-glyceric acid

Phase IV. Phosphoglyceric acid is split into pyruvic acid and phosphoric acid



Phase V. Pyruvic acid is reduced to lactic acid at the expense of the oxidation of glycerophosphoric acid to triosephosphoric acid



The triosephosphoric acid is further changed (III and V), thus the process repeats itself.

Intermediary Products of Alcoholic Fermentation. Scheme of Meyerhof. Meyerhof and associates (42) suggested a scheme based on results obtained by partial inhibition of the zymase-cozymase complex, and the isolation of the various intermediary products. The inhibitors used were sodium fluoride and monoiodoacetic acid. The reactions were divided into three groups, of which the first two (*a* and *c*) were inhibited by iodoacetate but not by fluoride. The third group was inhibited by fluoride but not by iodoacetate (*b*).

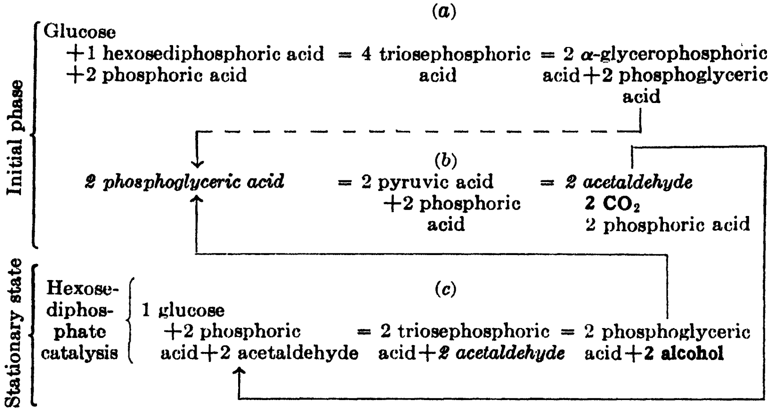
(*a*) 1 Glucose + 1 hexosediphosphoric acid + 2 phosphoric acid = 2 α-glyceric phospheric acid + 2 phosphoglyceric acid.

(*b*) Phosphoglyceric acid = pyruvic acid + phosphoric acid = acetaldehyde + CO_2 + phosphoric acid.

(*c*) 1 Glucose + 2 phosphoric acid + 2 acetaldehyde = 2 alcohol + 2 phosphoglyceric acid.

(hexosediphosphate catalysis)

Scheme of Alcoholic Fermentation (Meyerhof)


Initial phase
(a)
Glucose
+1 hexosediphosphoric acid = 4 triosephosphoric acid = 2 α-glycerophosphoric acid+2 phosphoglyceric acid
+2 phosphoric acid
(b)
2 phosphoglyceric acid = 2 pyruvic acid +2 phosphoric acid = 2 acetaldehyde 2 CO₂ 2 phosphoric acid
Stationary state
Hexose-diphosphate catalysis
(c)
1 glucose +2 phosphoric acid+2 acetaldehyde = 2 triosephosphoric acid+2 acetaldehyde = 2 phosphoglyceric acid+2 alcohol

This scheme covers all phases of alcoholic fermentation.

By comparing the scheme of lactic acid fermentation with that of alcoholic fermentation, it may be seen that the two reactions are in close relationship.

REFERENCES

1. Harden, A.: Alcoholic fermentation. The early stages of fermentation. Fermentation in the yeast cell. *Ergebnisse Enzymforschung*, **1**, 113 (1932).
2. Meyerhof, O.: Über die enzymatische Milchsäurebildung im Muskelextrakt (Dritte Mitteilung: Die Milchsäurebildung aus den gärfähigen Hexosen). *Biochem. Z.*, **183**, 176 (1927).
3. Lohmann, K.: Über das Koferment der Milchsäurebildung des Muskels. *Naturwissenschaften*, **19**, 180 (1931).
4. Erdtman, H.: Über Nierenphosphatase und ihre Aktivierung. II. *Z. physiol. Chem.*, **177**, 211 (1928).
5. Kay, H. D.: Plasma phosphatase. I. Method of determination. Some properties of the enzyme. *J. Biol. Chem.*, **89**, 235 (1930).

6. Neuberg, C., and Hildesheimer, A.: Über zuckerfreie Hefegärungen. I. *Biochem. Z.*, **31**, 170 (1911).
7. Anhagen, E.: Co-Carboxylase, ein neues Co-Enzym der alkoholischen Gärung. *Z. physiol. Chem.*, **204**, 149 (1932).
8. Lebedew, A., and Griaznoff, N.: Über den Mechanismus der alkoholischen Gärung. II. *Ber.*, **45**, 3256 (1912).
9. Willstätter, R., and Steibelt, W.: Bestimmung der Maltase in der Hefe. *Z. physiol. Chem.*, **111**, 157 (1920).
10. Willstätter, R., and Oppenheimer, G.: Über Lactasegehalt und Gärvermögen von Milchzuckerhefen. *Z. physiol. Chem.*, **118**, 168 (1922).
11. Willstätter, R., and Lowry, C. D.: Invertinverminderung in der Hefe. Elfte Abhandlung zur Kenntnis des Invertins. *Z. physiol. Chem.*, **150**, 287 (1925).
12. Myrbäck, K.: Die Co-Zymase und ihre Bestimmung. *Z. physiol. Chem.*, **177**, 158 (1928).
13. Meyerhof, O.: Über das Gärungscoferment in Tierkörper (Zweite Mitteilung). I. Die Verbreitung des Coferments in Tierkörper. *Z. physiol. Chem.*, **102**, 3 (1918).
14. Euler, H. von, and Myrbäck, K.: Gärungs-Co-Enzym (Co-Zymase) der Hefe. II. *Z. physiol. Chem.*, **133**, 260 (1924).
15. Euler, H. von, and Myrbäck, K.: Gärungs-Co-Enzym (Co-Zymase) der Hefe. I. 2. Co-Zymasepräparate. *Z. physiol. Chem.*, **131**, 182 (1923).
16. Oppenheimer, C., and Pincussen, L.: Methodik der Fermente. Die Fermente und ihre Wirkung. Bd. III. 5 Aufl. Thieme (1928).
17. Willstätter, R., and Steibelt, W.: Über die Gärwirkung maltasearmer Hefen (Vierte Mitteilung über Maltase). *Z. physiol. Chem.*, **115**, 211 (1921).
18. Nilsson, R.: Studies upon the enzymic degradation of carbohydrates. *Arkiv Kemi*, **10**, A, No. 7 (1930).
19. Meyerhof, O.: Über das Gärungscoferment in Tierkörper (Zweite Mitteilung). I. Die Verbreitung des Coferments in Tierkörper. *Z. physiol. Chem.*, **102**, 3 (1918).
20. Harden, A., and Young, W. J.: The alcoholic ferment of yeast-juice. *J. Physiol.*; *Proc.*, Nov. 12, 1904, **32**, 1.
21. Harden, A., and Young, W. J.: The influence of phosphates on the fermentation of glucose by yeast juice (preliminary communication). *Proc. Chem. Soc. London*, **21**, 189 (1905).
22. Euler, H. von, and Myrbäck, K.: Gärungs-Co-Enzym (Co-Zymase) der Hefe. I. *Z. physiol. Chem.*, **131**, 179 (1923).
23. Meyerhof, O.: Über das Vorkommen des Coferments der alkoholischen Hefegärung im Muskelgewebe und seine mutmassliche Be-

deutung im Atmungsmechanismus. *Z. physiol. Chem.*, **101**, 165 (1917–18).

24. Meyerhof, O.: Die Energieumwandlungen im Muskel. Kapitel V. Beeinflussungen von Milchsäurebildung und Säuerstoffatmung. *Pflügers Arch.*, **188**, 114 (1921).
25. Lohmann, K.: Über die Pyrophosphatfraktion im Muskel. *Naturwissenschaften*, **17**, 624 (1929).
26. Euler, H. von, and Myrbäck, K.: Co-Zymase und Adenylsäure. *Z. physiol. Chem.*, **199**, 189 (1931).
27. Nilsson, R., and Euler, H. von: Co-Zymase und Adenosintriphosphat. *Z. physiol. Chem.*, **204**, 204 (1932).
28. Euler, H. von, and Nilsson, R.: Adenosintriphosphorsäure und Co-Zymase. *Z. physiol. Chem.*, **208**, 173 (1932).
29. Meyerhof, O., and Lohmann, K.: Über das Co-Fermentsystem der Milchsäurebildung. *Naturwissenschaften*, **20**, 387 (1932).
30. Euler, H. von, Adler, E., Schlenk, F., and Günther, G.: Über die Komponenten der Dehydrasesysteme. V. Die Wirkungen der Co-Zymase, des Warburgschen Co-Fermentes und des Adenylpyrophosphates. *Z. physiol. Chem.*, **233**, 120 (1935).
31. Myrbäck, K.: Co-Zymase. *Ergebnisse Enzymforschung*, **2**, 154 (1933).
32. Myrbäck, K., and Euler, H. von: Über die chemische Natur der Co-Zymase. *Z. physiol. Chem.*, **203**, 143 (1931).
33. Myrbäck, K., and Örtenblad, B.: Zur Kenntnis der Co-Zymase. IV. *Z. physiol. Chem.*, **233**, 87 (1935).
34. Myrbäck, K.: Zur Kenntnis der Co-Zymase. V. *Z. physiol. Chem.*, **233**, 95 (1935).
35. Warburg, O., Christian, W., and Griese, A.: Wasserstoffübertragendes Co-Ferment, seine Zusammensetzung und Wirkungsweise. *Biochem. Z.*, **282**, 157 (1935).

35*a*. Euler, H. von, Albers, H., and Schlenk, F.: Über die Cozymase. *Z. physiol. Chem.*, **237**, I; (1935); **240**, 113 (1936).

35*b*. Harden, A., and Henly, F. R.: The equation of alcoholic fermentation. *Biochem. J.*, **21**, 1216 (1927).

35*c*. Harden, A., and Henly, F. R.: The equation of alcoholic fermentation. II. *Biochem. J.*, **23**, 230 (1929).

36. Kluyver, A. J., and Struyk, A. P.: The first phases of the chemistry of the dissimilation of the hexoses. II. *Proc. Acad. Amsterdam*, **31**, 882 (1928).
37. Harden, A., and Young, W. J.: The alcoholic ferment of yeast-juice. Part III. The function of phosphates in the fermentation of glucose by yeast-juice. *Proc. Roy. Soc.* (*B*), **80**, 299 (1908).
38. Harden, A. (quoted by): Alcoholic fermentation. The early stages

of fermentation. Fermentation in the yeast cell. *Ergebnisse Enzymforschung*, **1**, 112 (1931).

39. Robison, R.: A new phosphoric ester produced by the action of yeast juice on hexoses. *Biochem. J.*, **16**, 809 (1922).
40. Robison, R., and King, E. J.: Hexosemonophosphoric esters. *Biochem. J.*, **25**, 323 (1931).
41. Embden, G., Deuticke, H. J., Kraft, G.: Über die intermediären Vorgänge bei der Glycolyse in der Musculatur. *Klin. Wochenschr.* **12**, 213 (1932).
42. Meyerhof, O., and Kiesling W.: Über die phosphorylierten Zwischenprodukte und the letzten Phasen der alkoholischen Gärung. *Biochem. Z.*, **267**, 313 (1933).

CHAPTER X

CARBONIC ANHYDRASE

The general belief until recently was that the blood CO_2 is carried mainly in the form of bicarbonate, and when the blood reaches the lungs, evolution of CO_2 takes place, according to the following reactions:

$$\text{HP (= weak protein acids of blood)} + NaHCO_3 \rightarrow \text{NaP (protein salt)} + H_2CO_3$$

$$H_2CO_3 \rightarrow CO_2 + H_2O$$

In 1926 Henriques (1) calculated the rate at which CO_2 could be formed at *p*H 8, and found that it could be much less than that observed in the expired air (2).

In view of this, he believed that a catalyst for the reaction $H_2CO_3 \rightleftharpoons CO_2 + H_2O$ must be present in the blood, or CO_2 transportation must be due to a mechanism other than the bicarbonate one. Although Henriques furnished some fundamentally important experiments, he could not show that the action is caused by an enzyme or catalyst. In 1930, experiments of Hawkins and Van Slyke (3) pointed directly to a catalytic effect, and in 1932, Meldrum and Roughton (4, 5) isolated from red blood cells a highly active enzyme which they called carbonic anhydrase.

Preparation of the Enzyme

A Preparation of the Crude Enzyme. One hundred cubic centimeters of ox red cells is placed in a centrifuge tube and washed three times with normal saline; 80 cc. of H_2O, 20 cc. of ethyl alcohol, and 150 cc. chloroform are added and shaken at room temperature for five minutes. The mixture is allowed to stand over night at 2°. After ten minutes of centrifuging at 3500 r.p.m., the third layer which separates on the top contains the enzyme. This solution when dried in vacuum has a brown color and contains methemoglobin and related substances, as well as much catalase. The yield is 1 gram per 100 cc. blood cells and contains 50 per cent of all activity present in the cells. The dry preparation is water-soluble. Aqueous solutions keep for weeks in the ice chest.

Preparation of Purified Enzyme. First the dialyzable impurities are removed by ultra-filtration of the third layer through Bechhold's 6 per cent and 4.5 per cent ultra-filtration membranes. The ultra-filtration may be carried out by vacuum suction, provided the fluid on the membrane is stirred. Now the non-dialyzable impurities are removed by three treatments of an equal volume of $Al(OH)_3$(C-γ alumina cream according to Willstätter). The final solution left after adsorption has an activity of 1730 units per mg. This final activity is 400 times that of the original red cells. This means that 1 part of the pure solid in 7,000,000 parts of solution is sufficient to double the rate of CO_2 evolution, and that during the first fifteen seconds 1 gram of enzyme liberates 825 grams of CO_2 or it can produce 1.24 mols of CO_2 per second, per gram of enzyme.

Non-dialyzable impurities may also be removed by using $Ca_3(PO_4)_2$ suspension instead of $Al(OH)_3$.

Method for the Estimation of Activity

The catalytic effect upon the rate of CO_2 evolution from sodium bicarbonate solution when mixed with phosphate buffer of pH 6.8 is measured. In one compartment of the glass boat-shaped trough (Fig. 28) is placed 2 cc. of phosphate solution (equal volume of $M/5$ Na_2HPO_4 and $M/5$ KH_2PO_4), whereas in the other compartment, 2 cc. of $M/5$ $NaHCO_3$, dissolved in $M/50$ NaOH is placed. The boat is attached by a stopper and pressure tubing to a manometer, open at the other end to the air. Time is allowed for temperature equilibration, and the boat is shaken rapidly in a constant-temperature water bath of 15°. The evolution of CO_2 is followed by observing the reading of the manometer at 0, 5, 10, 15, 30, 45, 60, 90, and 120 seconds and thereafter at minute intervals if required. If the first half of the total CO_2 is evolved within 15 seconds, diffusion from the liquid to gas phase is not a limiting factor. The rate of CO_2 evolution as

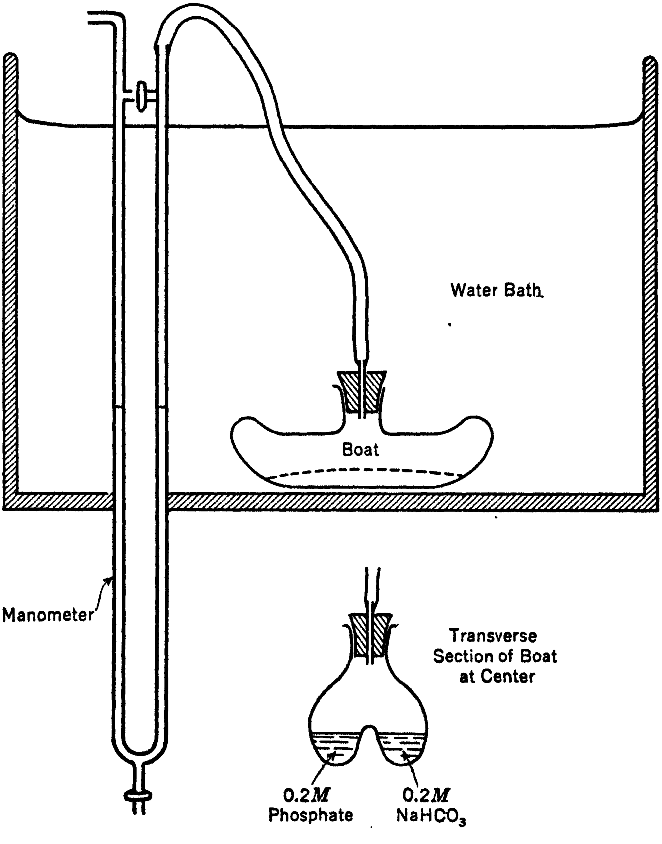


FIG. 28.—Boat technic for measurement of enzyme activity

observed is a true measure of the velocity of the chemical reaction $H_2CO_3 \rightarrow CO_2 + H_2O$.

The Enzyme is Specific

The purified preparation shows no catalase, peroxidase, or oxidase action, and when spectrometrically tested in thick layers, it gives no indication of hemoglobin or hematin compounds (4, 6).

Chemical Nature

The purified dry enzyme has a white color. It gives all the protein tests and a slight Molisch test. Prolonged boiling of aqueous solutions destroys it. It does not dialyze through membranes readily. The kinetics of this enzyme has not been extensively studied as yet. Buffered solutions from *p*H 4 to *p*H 11 keep for thirty minutes. At *p*H 3 and 13, however, there is complete destruction. The enzyme is poisoned by CO, HCN, sulfides, azides, Cu, Ag, Au, Zn, and phenylurethane.

The amount of carbonic anhydrase in blood during certain pathological conditions and its presence in various organs deserve consideration.

REFERENCES

1. HENRIQUES, O. M.: Die Bindungsweise des Kohlendioxyds im Blute. *Biochem. Z.*, **200**, 1 (1928).
2. FAURHOLT, C.: Studies on aqueous solutions of carbonic anhydride and carbonic acid. Also: Studies of aqueous solutions of carbamates and carbonates. *J. chim. phys.*, **21**, 400 (1924); **22**, 1 (1925).
3. VAN SLYKE, D. D., and HAWKINS, J. A.: Studies of gas and electrolyte equilibria in blood. XVI. The evolution of carbon dioxide from blood and buffer solutions. *J. Biol. Chem.*, **87**, 265 (1930).
4. MELDRUM, N. U., and ROUGHTON, F. J. W.: The CO_2 catalyst present in blood. *J. Physiol.*, **75**, 4 (1932). Some properties of carbonic anhydrase, the CO_2 enzyme present in blood. *Ibid.*, **75**, 15 (1932).
5. ROUGHTON, F. J. W.: Carbonic anhydrase. *Ergebnisse Enzymforschung*, **3**, 289 (1934).
6. STADIE, C. WM., and O'BRIEN, H.: The catalysis of the hydration of carbon dioxide and dehydration of carbonic acid by an enzyme isolated from red blood cells. *J. Biol. Chem.*, **103**, 521 (1933).

CHAPTER XI

LUCIFERASE

The enzyme luciferase was discovered in 1885 by Dubois in the elaterid beetle *Pyrophorus noctilucus* (1) and the mollusc *Pholas dactylus* (2). Harvey (3) showed that luciferase is present in fireflies, in ostracod crustaceans (4), and in the worm *Odontosyllis* (5), and it has been found in the fish *Malacocephalus* by Hickling (6). According to Harvey (7), who has contributed most to the knowledge of this enzyme, it occurs in forty different orders of animals, certain classes of plants, and in bacteria and fungi. The firefly, however, is the best-known luminous animal. Some familiar examples of bioluminescence are the glowing of wood, the phosphorescence of the sea, and the shining of fish. They are all due to microscopic organisms. Bioluminescence is a distinct form of chemiluminescence, resulting from the energy change in a chemical reaction.

Luciferin, the substrate of luciferase, was also discovered by Dubois (1, 2). Luciferin is a rare example of a substrate with an unknown chemical nature. The oxidation product of luciferin (after luciferase has been acted upon) has been named oxiluciferin by Harvey (8). The luciferin differs slightly with the species, and the luciferase acts only with luciferin of closely related animals (5, 9).

Preparation (7). Luciferase may be prepared from either fresh or dried luminous material by cold water extraction. It is best to have the material stand in cold water until all luciferin is oxidized. The oxidation may be hastened by the addition of chloroform, saponin, or sodium glycocholate—compounds which aid in the liberation of luciferin, apparently owing to cytolysis.

Luciferin may be obtained by adding hot water to the luminous tissue or by rapidly heating the luminescent extract to "temperatures which permanently quench the light" or to the boiling point. This procedure destroys the luciferase before much of the luciferin is oxidized. Luciferin is heat stable, but the solution must be cooled quickly so as to prevent oxidation of the luciferin.

Purification methods for luciferase have been described by Harvey (10) and by Kanda (11).

Mechanism of Luciferase Action

Luciferin in the presence of luciferase produces light. No light appears, however, on non-enzymic oxidation of luciferin. When luxiferin is oxidized, oxyluciferin forms: LH_2 (luciferin) + $\frac{1}{2}O_2$ = L (oxyluciferin + H_2O), which may again be reduced to luciferin: L (oxyluciferin + H_2 = LH_2 (luciferin). Nascent hydrogen may be used for the reduction (8, 12). The luciferin-oxyluciferin system is similar to the leuco methylene blue-methylene blue system and other reversibly oxidizable dyes (13).

Chemical Nature of Luciferin

Cypridina luciferin dialyzes through membranes. It is not destroyed by trypsin and is soluble in water, in many organic solvents, in diluted salt, acid, and alkali. In alkaline medium it oxidizes readily, but keeps for years in aqueous solution. Saturated $(NH_4)_2SO_4$ precipitates the luciferin but not $MgSO_4$ or NaCl (10, 14). In view of these results, it may be said that the chemical nature of luciferin is unknown. Kanda (15) reported the crystallization of luciferin. No further details, however, are presented in his report.

Chemical Nature of Luciferase

Cypridina luciferase does not dialyze through membranes; it is destroyed by trypsin; insoluble in practically all organic solvents; soluble in water, diluted salt solutions, diluted acid, and alkali. Protein precipitants precipitate the enzyme (10, 11). In contrast with luciferin, luciferase forms an antiluciferase when injected into the blood of rabbits (16). These properties of luciferase point to a protein nature.

Methods for the Estimation of Luciferase Activity

The direct photometric method (7), the photoelectric procedure (17), or the photographic principle may be employed (18).

The kinetics of this enzyme has been discussed by Harvey (7).

REFERENCES

1. Dubois, R.: Fonction photogenique des pyrophores. *Compt. rend. soc. biol.*, Ser. 8, **2**, 559 (1885).
2. Dubois, R.: Note sur la fonction photogenique chez les pholades. *Compt. rend. soc. biol.*, Ser. 8, **4**, 564 (1887).
3. Harvey, E. N.: The mechanism of light production in animals. *Science*, **44**, 208 (1916).
4. Harvey, E. N.: Studies on bioluminescence. IV. The chemistry of light production in a Japanese ostracod crustacean, *Cypridina hilgendorfii*, Müller. *Am. J. Physiol.*, **42**, 318 (1916–17).
5. Harvey, E. N.: Studies on bioluminescence. XIV. The specificity of luciferin and luciferase. *J. Gen. Physiol.*, **4**, 285 (1921–22).
6. Hickling, C. F.: A new type of luminescence in fishes. *J. Marine Biol. Assoc. United Kingdom*, **13**, 914 (1925).
7. Harvey, E. N.: Luciferase, the enzyme concerned in luminescence of living organisms. *Ergebnisse Enzymforschung*, **4**, 365 (1935).
8. Harvey, E. N.: Studies on bioluminescence. VII. Reversibility of the photogenic reaction in *Cypridina*. *J. Gen. Physiol.*, **1**, 133 (1918–19).
9. Harvey, E. N.: Additional data on the specificity of luciferin and luciferase, together with a general survey of the reaction. *Am. J. Physiol.*, **77**, 548 (1926).
10. Harvey, E. N.: Studies on bioluminescence. IX. Chemical nature of *Cypridina* luciferin and *Cypridina* luciferase. *J. Gen. Physiol.*, **1**, 269 (1919).

11. Kanda, S.: Physico-chemical studies on bioluminescence. IV. The physical and chemical nature of the luciferase of *Cypridina hilgendorfii*. *Am. J. Physiol.*, **55**, 1 (1921).
12. Harvey, E. N.: Studies on bioluminescence. XV. Electroreduction of oxyluciferin. *J. Gen. Physiol.*, **5**, 275 (1922–23).
13. Harvey, E. N.: The oxidation-reduction potential of the luciferin-oxyluciferin system. *J. Gen. Physiol.*, **10**, 385 (1926–27).
14. Kanda, S.: Physico-chemical studies on bioluminescence. V. The physical and chemical nature of luciferine of *Cypridina hilgendorfii*. *Am. J. Physiol.*, **68**, 435 (1924).
15. Kanda, S.: Crystalline luciferin. *Sci. Papers Inst. Phys. Chem. Research*, Suppl. **18**, 1 (1932).
16. Harvey, E. N., and Dietrick, J. E.: The production of antibodies for *Cypridina* luciferase and luciferin in the body of a rabbit. *J. Immunol.*, **18**, 65 (1930).
17. Anderson, R. S.: The chemistry of bioluminescence. I. Quantitative determination of luciferin. *J. Cell. Comp. Physiol.*, **3**, 45 (1933).
18. Anderson, W. R.: Kinetics of the bioluminescent reaction in *Cypridina*. I. *J. Gen. Physiol.*, **4**, 517 (1922).

AUTHOR INDEX

A

Abderhalden, E., 49, 50, 66, 90, 91, 94
Adams, M., 139, 140, 143
Adler, E., 173, 204, 214
Agner, K., 162
Ahlgren, 176
Akabori, S , 67, 68, 72
Akamatsu, S., 38
Albers, H., 214, 215
Albl, H., 186
Alsberg, C. L., 180
Ambros, O., 94, 109
Ammon, R., 14, 23, 32, 42, 44
Andersen, B., 57, 58, 65, 171, 172, 175
Anderson, R. S., 229
Anhagen, E., 211
Anson, M. L., 17, 65, 92
Armbruster, R., 36
Armstrong, E. F., 132, 135, 136
Arnovljevic, V., 94
Arrhenius, S., 2, 64
Aubry, A., 130
Avery, O. T., 147, 179
Avtonomova, E. S., 158, 159

B

Bach, A., 107, 108, 179
Baker, J. G., 142
Baker, Z., 14, 33
Baldwin, M. W., 7
Balls, A. K., 90, 93, 106, 179
Bamann, E., 7, 30, 38, 67, 130
Bancroft, G., 178
Banga, I., 171, 172, 203
Banshi, H., 183
Barendrecht, H. P., 113
Barnum, G. L., 190
Basu, K. P., 115
Bates, R. W., 67, 68
Batteli, F., 172, 176
Bauer, K., 147
Bayliss, W. M., 22, 66
Beber, M., 159
Bechhold, H., 1
Beguin, C., 135
Bender, R., 128, 147
Bergemann, O. H. K., 186
Bergmann, M., 50, 86, 87, 91, 92, 93, 94
Bernhauer, K., 175
Bernheim, F , 172, 176
Bernheim, M. L. C , 107, 176
Bersin, Th., 85
Bertrand, G., 136, 184, 186
Bierry, H , 131, 139
Birch, T. W , 171
Bloch, B., 185, 186
Blood, A. F., 94
Bodansky, A., 3, 38, 39
Bodansky, O., 9, 37
Bonem, P., 115
Booher, L. E., 18, 144
Borhardt, H., 128
Borsook, H., 22, 180, 208
Bourquelot, E., 130, 137, 147, 184
Boyland, E., 171
Boyland, M. E., 171
Bradley, H. C., 131
Brann, L., 160, 180
Bredereck, H., 136
Bridel, M., 135, 136
Broman, T., 173
Bronnicke-Funder, J., 65
Brücke, E., 17, 19
Burk, D., 133
Butterworth, J., 172

C

Cajori, F. A., 7, 131, 132, 134
Caldwell, M. L., 18, 139, 140, 142, 143, 144, 145
Cambier, P., 39
Carey, P. C., 187
Carmichael, E. A., 39
Carrie, C., 159
Chain, P., 23
Chibnall, A. C., 106
Chodat, R., 107, 179, 184
Chopra, R. N., 94
Christian, W., 19, 203, 204, 205, 208, 214, 215
Clark, E. D., 142
Clark, W. M., 112
Cleaveland, M., 139
Clifford, W. M., 59
Clift, F. P., 174, 175
Cohnheim, O., 88
Compton, A., 3
Cook, R. P., 174, 175
Csanyi, W., 7
Cullen, G. E., 113, 147

D

Dakin, H. D., 30, 33, 115, 179
Dale, H. H., 39
Dale, J. E., 140
Dambovicaeanu, A., 161
Damodaran, M., 106
Davidsohn, H., 7, 32, 127
Davies, D. R., 173
Davison, W. G., 13
Dernby, K. G., 8
Deuticke, H. J., 215
Dick, H., 21
Diedrichs, K., 38
Dixon, M., 170, 178, 187, 209
Dodonowa, E. W., 128
Doebbeling, S. E., 140, 142, 144, 145
Dooren, F. von, 39
Dubiski, 94
Dubois, R., 227
Dubourbfaut, 137
Dunaiturria, S., 65
Dyckerhoff, H., 6, 20, 36, 49, 90, 91, 93, 106

E

Eadie, G. S., 139, 140
Easson, L. H., 39, 64
Edlbacher, S., 115, 117, 118
Effront, J., 12
Ehrbacher, O., 130
Ehrenwall, E., 91, 94
Ehrlich, P., 187
Einbeck, H., 169
Elliott, K. A. C., 178, 179, 183
Elvove, E., 33
Ely, J. O., 39
Emden, G., 217
Erdtmann, H., 211
Euler, H. von, 8, 18, 21, 125, 127, 147, 157, 158, 159, 160, 170, 173, 174, 194, 204, 212, 213, 214, 215
Ewins, A. J., 107

F

Falk, K. G., 10, 14, 21, 30, 35
Faurholt, C., 223
Fearon, W. R., 108, 109
Feldberg, W., 39
Felix, K., 49
Fenton, H J. H., 108
Fernbach, A., 36
Fischer, E., 125, 128, 134, 135, 136
Fischgold, H., 44
Fleisch, A., 169
Fleury, P., 186
Florkin, M., 183
Foa, C., 131
Fodor, A., 20, 22
Folley, S. J., 38
Frage, K., 172
Fraser, F. R., 39
Freudenberg, E., 132
Freudenberg, K., 36
Fruton, J. S., 86, 87
Fuchs, B., 115

G

Geddes, W. F., 106
Gesenius, H., 49
Giaja, J., 131, 139
Gierisch, W., 161

Glick, D., 12, 14, 15, 19, 30, 32, 33, 34, 35
Goddard, D. R., 53
Gootz, R., 136
Gore, H. C., 142
Govaerts, P , 39
Gözsy, B., 171
Grabar, P., 111
Grant, G. A., 131
Grassmann, W., 6, 49, 66, 85, 87, 88, 90, 91, 93, 94, 106, 115, 128, 147
Green, J. R., 35
Greenstein, J. P., 52
Grover, C. E., 106
Günther, E., 136

H

Haehn, H., 139
Hahn, A., 140, 170
Hahn, H., 7
Haldane, J. B. S., 13
Hale, W. S., 179
Haley, D. E., 35
Halloway, R. G , 108
Hammarsten, O., 57
Hand, D. B., 108, 110, 179, 183
Hanes, C. S., 142
Happold, F. C., 185, 186
Harden, A., 211, 213, 215
Hare, M. L. C., 107, 179
Harmann, W., 170
Harrison, D. C., 168, 172, 174, 175, 176, 193
Harteneck, A., 49, 66
Harvey, E. N., 227, 228, 229
Hauge, S. M., 190
Hawkins, J. A., 223
Haworth, W. N., 146
Hedin, S. G., 58
Heidenhain, R., 66
Heiss, H., 180
Helferich, B., 6, 7, 134, 135, 136
Hellerman, L., 112
Hellström, H., 157, 158, 160, 161
Helmert, E., 85
Henly, F. R., 215
Hennichs, S., 160
Henseleit, K., 114
Herriott, R. M., 54, 63
Hesse, A. R., 18, 143
Heyde, W., 86
Heyroth, F. F., 53
Hickling, S. F., 227
Hildebrand, F. C., 142
Hildesheimer, A., 211
Hill, R., 187
Hintner, H., 42
Hirsch, P., 21
Hizume, K., 137
Hoffmann, P., 132
Hofmann, E., 131
Holmberg, C. G., 170, 171
Holter, H., 18, 53, 57, 58, 65
Hopkins, F. G., 174, 190
Hoppe-Seyler, F., 193
Hoppert, C , 114
Hosokawa, T., 111
Hotchkiss, M., 128
Howell, S. F., 4, 7, 21, 111, 113, 114, 127, 183
Hoyer, E , 35
Hudson, C. S., 125, 135, 138
Hug, E., 36
Hull, M., 21
Hunt, R., 39
Hunter, A., 106
Husfeldt, E., 86

I

Inouye, K., 38
Ishiyama, T., 5
Ivanoff, N. N., 108, 128

J

Jacoby, M., 108, 111
Jaffe, H. L., 38, 39
Jeffreys, C. E. P., 180
Johnson, A., 21
Johnston, M. E., 33
Josephson, K., 18, 21, 127, 136, 157, 158, 159, 160
Jozsa, S., 142

K

Kahn, J., 186
Kanda, S., 228, 229
Karrer, P., 147

Karstroem, H., 128
Kastle, J. H., 4, 33
Katschioni-Walther, L., 49
Kay, H. D., 7, 37, 38, 39, 211
Keilin, D., 168, 169, 170, 179, 187, 193
Kendall, E. C., 142
Kiesling, W., 218
King, C. G., 12, 13, 14, 15, 19, 30, 33, 34
King, E. J., 216
Kirk, J. S., 21, 111, 112, 113
Kitagawa, M., 111
Kjeldahl, M. J., 125
Klein, G., 117
Kleiner, I. S., 6, 7, 18, 20, 21, 53, 54, 55, 59, 69, 81, 94, 111, 113, 128, 129, 130, 134, 161, 162, 188, 189, 190
Kleinmann, H., 86
Klinkenberg, van, G. A., 138
Kluyver, H. J., 215
Knaffl-Lenz, E., 15
Koch, F. C., 67
Kochmann, R., 111
Kocholaty, W., 117
Kodama, K., 179
Kofranyi, E., 20
Kogh, T., 186
Köhler, F., 37, 90, 93
Köller, F., 118
Komm, E., 50
Kossel, A., 115
Kraft, G., 217
Kraus, J., 117, 118
Kraut, H., 33, 130
Krebs, H. A., 2, 86, 114, 176
Kröber, E., 4
Kröner, W., 49
Kuhn, R., 6, 7, 13, 19, 33, 127, 129, 134, 138, 139, 160, 180, 183, 207, 208
Kühne, W., 66, 88
Kullberg, S., 125
Kunitz, M., 15, 18, 24, 69, 71, 72, 73, 76, 80, 81
Künstner, G., 49, 65
Kusenack, W., 146

L

Laeverenz, P., 30
Laidlaw, P., 107
Laki, K., 172
Lappe, J., 131
Larson, H. W., 11
Lebedew, A., 212
Lee, E. R., 7
Lehmann, J., 172, 173
Leibowitz, J., 129, 146
Leinert, F., 91
Leuthardt, F., 118
Lewitow, M., 131
Lind, O., 33
Lindberg, E., 125
Linderstroem-Lang, K., 58, 65, 66, 68, 106
Lindner, P., 147
Lineweaver, H., 133
Linhardt, K., 114
Lintner, C. J., 4
Loeb, L., 112
Loew, F., 128
Loewenhart, A. S., 4, 33
Logemann, W., 85
Lohmann, J., 172, 173
Long, J. A., 21
Longenecker, H. E., 35
Loughlin, W. H., 18
Lövgren, S., 113, 114
Lowry, C. D., 212
Luck, J. M., 109
Lüers, H., 4, 18, 144
Lundsgaard, E., 175
Lutz, J. G., 126, 127

M

MacMunn, C. A., 193
Majorow, S., 108
Makov, A. M., 161
Mangold, E., 94
Mann, P. V. G., 171, 175
Märcker, 137
Martland, M., 7
Maschmann, E., 85, 94
Matavulf, P., 94
Matsui, J., 51

Matsuyama, M., 159
Maximowitsch, S. M., 158, 159
Mayr, O., 106
McCance, R. A., 185
McLeod, J., 179
Mechlinski, P., 129
Melander, K., 125
Meldrum, N. U., 223, 226
Memmen, F., 32, 33
Mendel, L. B., 94
Menten, M. L., 10, 11, 12, 13
Merrill, H. B., 12, 13
Meyer, H., 140
Meyerhof, O., 171, 175, 211, 212, 213, 214, 216, 218
Michaelis, L., 7, 10, 11, 12, 13, 22, 32, 53, 64, 125, 130, 140, 158, 161
Michalik, R., 140
Michlin, D., 131
Miekeley, A., 93
Mirsky, A. E., 17, 65
Mishkind, D., 21, 182
Mitchell, W., 174
Mizusawa, H., 172
Morgan, E. J., 174, 190
Morgulis, S., 158, 159
Müller, D., 173
Mulzer, P., 186
Münch, H., 109, 129
Murray, D. R. P., 13, 14
Myrbäck, K., 111, 128, 140, 170, 212, 213, 214

N

Nasse, O., 139
Needham, D., 176
Nelson, J. M., 8, 11, 126, 127
Neuberg, C., 36, 114, 134, 186, 211
Nichols, J. B., 53
Niebel, W., 128
Nilsson, R., 170, 174, 213, 214
Noguchi, J., 114
Northrop, J. H., 7, 9, 10, 12, 15, 16, 18, 19, 20, 21, 22, 52, 53, 54, 56, 58, 60, 63, 64, 65, 68, 69, 71, 72, 73, 76, 96, 81
Nosaka, K., 159
Noyes, H. M., 10

O

O'Brien, H., 226
Ohlsson, E., 138, 169
Onslow, M. W., 179
Onslow-Wheldale, M., 186
Oppenheimer, G., 7, 130, 132, 134, 212
Ortenblad, B., 214
Osborne, T. B., 49
O'Sullivan, C., 125
Oswald, W., 21

P

Pace, J., 67, 69
Palmer, L. S., 13
Parastschuk, S. W., 16, 17
Pavlov, J. P., 16, 17
Pavlovic, R., 13
Pechstein, H., 140, 158, 161
Peck, S. M., 186
Pekelharing, C. A., 18, 60, 64
Perkins, M. E., 112
Perrin, J., 19
Peters, O., 136
Philoche, C., 137
Pierce, G., 33
Pincussen, L., 212
Plattner, F., 42
Pohl, J., 187
Poland, L. O., 112
Pollinger, A., 180, 183
Porcher, C., 131
Portier, P., 131, 186
Pottevin, H., 36
Pringsheim, H., 128, 146, 147
Pugh, C. E. M., 180, 185
Purr, A., 85, 87, 92, 117, 140, 141

Q

Quastel, J. H., 107, 112, 170, 173

R

Rabkin, I., 159
Racke, F., 126, 127
Raper, H. S., 180, 185
Reichel, M., 18, 138, 144
Renshaw, R. R., 39
Rhind, D., 36

Richardson, G. M., 50
Riegert, A., 111
Rigoni, M., 108
Roberts, W. M., 38, 39
Robinson, M. B., 185
Robinson, R., 7
Robison, M. E., 179, 216
Roche, J., 38
Roe, J. H., 190
Rohdewald, M., 19, 53, 67, 68, 139
Röhmann, F., 131
Rona, P., 13, 14, 23, 32, 44, 86, 130, 161
Rosenfeld, B., 179
Rosenfeld, L., 36, 112
Rosenthal, O., 114
Roughton, F. J. W., 223, 226
Roy, A. C., 94
Rubenbauer, H., 33
Rudy, H., 207, 208
Russel, M., 85, 87, 116

S

Sabolitschka, T., 137
Salaskin, S., 116, 117
Salazar, G., 131
Sato, M., 93
Schaff, F., 186
Schäffner, A., 49, 66, 85, 88, 116, 117
Schardinger, F., 174
Scharikova, A., 85, 116, 117
Scharr, G., 86
Schepowalnikow, N. P., 66
Schleich, H., 91, 92, 93, 94
Schlenk, F., 214, 215
Schlesinger, M. D., 7, 143
Schmalfuss, H., 186
Schmalfuss, Z., 186
Schmidt, E. G., 111, 118
Schmiedeberg, O., 114
Schmitt, V., 93
Schmitz, H., 147
Schmitz-Hillebracht, E., 136
Schneider, K., 126
Schneider, L., 171
Schoenebeck, O., von, 91
Schönfeld-Reiner, R., 11
Schoonover, J. W., 39
Schreus, H. T., 159
Schubert, M. P., 11
Schubert, P., 147
Schulz, F. W., 94
Schütz, E., 9, 10
Schwab, E., 50, 66, 90
Schwann, T., 53
Schweigart, H., 139
Seifert, K., 146
Sellner, E., 144
Sen, K. C., 170, 174
Senter, G., 158
Seth, T. N., 109
Sherman, H. C., 7, 18, 139, 140, 142, 143, 144
Shibata, K., 51
Simons, E., 49
Sjögren, B., 53
Smirnowa, M. I., 108
Smith, F. E., 36, 38
Smith, J. C., 187
Smorodinzew, A., 114
Sobotka, H., 19, 33, 35, 134, 186
Solowjew, L., 116, 117
Sonderhoff, R., 172
Sørensen, S. P. L., 7, 49, 64, 159
Stade, C. Wm., 226
Stadler, R., 128, 147
Stamm, A. J., 53
Stankovic, R., 94
Starling, E. H., 66
State, W., 32
Staub, M., 147, 184
Staudinger, H., 146
Stedman, E., 39, 42
Steenberg, E. M., 68
Steibelt, W., 130, 212, 213
Steigerwaldt, F., 109
Steppuhn, O., 108
Stern, K. G., 2, 11, 22, 159, 160, 161, 162, 164
Stern, L., 170, 172, 180, 187
Stewart, C. P., 174
Stoeklin, E. D., 180
Stoll, A., 35, 36, 183
Struyk, A. P., 215
Sugiura, K., 10

Sumi, M., 108
Sumner, J. B., 4, 7, 19, 20, 21, 108, 109, 110, 111, 112, 113, 114, 127, 183
Suminskura, K., 186
Sundberg, C., 19
Sutter, H., 183
Svanberg, O., 127
Svedberg, Th., 53
Sym, E. A., 42
Szent-Györgyi, A. von, 2, 169, 171, 172, 185, 188, 203

T

Takahashi, H., 38
Takahata, T., 108
Takehiko, S., 114
Tanko, B., 36
Tapernoux, A., 131
Tarnanen, J., 106, 107
Tauber, H., 1, 6, 7, 15, 18, 20, 21, 23, 53, 54, 55, 56, 58, 59, 69, 81, 84, 94, 109, 110, 111, 112, 113, 128, 129, 130, 134, 136, 161, 162, 180, 182, 183, 188, 189, 190
Tawes, G., 20
Tazawa, Y., 51
Tenner, H. D., 39
Theorell, H., 205, 206, 207, 208
Thomas, A. W., 7
Thunberg, T., 168, 169, 170, 172, 173, 176
Thurlow, S., 172, 179
Tillmans, J., 21
Tompson, F. W., 125
Toos, B., 147
Toth, G., 128
Trurnit, H. I., 23
Tschastuchin, W. J., 128
Tyler, M. G., 140

U

Ucko, H., 183
Ulkin-Ljubowzoff, X., 108

V

Van Slyke, D. D., 65, 113, 114, 223
Vargha, L., 171
Vernon, H. M., 66
Vickery, H. B., 49
Vines, S. H., 84
Virtanen, A., 106, 107, 141
Vosburgh, W. C., 127
Voss, G., 42

W

Waentig, P., 161
Waksman, S. A., 13
Waldschmidt-Graser, J., 67
Waldschmidt-Leitz, E., 18, 20, 32, 35, 49, 65, 66, 67, 68, 72, 85, 88, 90, 92, 106, 109, 116, 117, 141, 142, 143, 144
Walker, T. K., 172
Walter, G., 117
Warburg, O., 19, 160, 168, 169, 187, 203, 204, 205, 208, 214, 215
Wasmund, W., 4
Wasserman, W., 126, 160
Wasteneys, H., 22
Weber, H., 183
Weber, H. H., 14, 49
Weholi, W., 147
Weidenhagen, R., 127, 128, 129, 135, 136, 138, 142
Weil, L., 115, 116, 117, 118
Weinstein, S. S., 32, 35
Whetham, M. D., 4, 170
White, A. C., 39, 42
Wieland, H., 161, 167, 172, 174, 179
Wigglesworth, V. B., 7, 132
Williams, J., 159
Willstätter, R., 7, 13, 19, 32, 33, 35, 36, 37, 53, 65, 66, 67, 68, 85, 87, 88, 94, 126, 127, 130, 134, 139, 142, 143, 180, 183, 212, 213
Winkler, S., 136
Wishart, G. M., 171, 176
Wohlgemuth, J., 142
Wolff, A., 138
Wolff, J., 180
Woodhouse, D. L., 30
Woolf, B., 107
Wynne, A. M., 32, 35

Y

Yamasaki, E., 108, 158, 159

YANOVSKY, E., 138
YOUNG, W. J., 213, 215

Z

ZACHARIAS, G., 114
ZECHMEISTER, L., 128
ZEHENDER, F., 85
ZEILE, K., 157, 158, 159, 160, 161
ZEISE, W., 117
ZERVAS, L., 86, 87, 91, 92, 93
ZUMSTEIN, O., 91

SUBJECT INDEX

A

Activation of enzymes, 12, 15, 30, 33, 34, 37, 53, 54, 66–68, 73, 77, 87, 89, 94, 111, 112, 116, 136, 141, 171, 180, 183, 211–213, 215
Adsorption of enzymes, 23, 33, 66, 67, 88, 90, 109, 116, 126, 130, 134, 136, 144, 157, 224
Alcohol dehydrogenase, 172
Alcoholic fermentation, 211
Aldehyde dehydrogenase, 174
Alpha-amylase, 138, 141, 144, 145, 146
Alpha-*d*-glucosidases, 129
Amidases, 106–119
Amino acid dehydrogenase, 176
Amylase, 137–146
  activators, 139, 140, 141
  chemical nature, 143, 144
  effect of ascorbic acid, 140
  effect of salts, 139, 140
  kinetics, 142, 145
  lyo- and desmo-amylases, 139
  optimum *p*H, 139–141
  separation of alpha- and beta-malt amylase, 144
  specificity, 139–141
  theories of starch decomposition, 138
Arginase, 115
  activation and estimation, 116–118
  in cancer, 116
  preparation, 115, 116
Ascorbic acid dehydrogenase, 190
Ascorbic acid oxidase, 188–190
  estimation, 189
  optimum *p*H, 189
  properties, 188
Asparaginase, 106
Aspartase, 107

B

Beta-amylase, 138, 140, 141, 144–146
Beta-glucosidase, 135
Beta-*d*-galactosidase, 131–134
Beta-hydroxybutyric dehydrogenase, 171
Biochemical catalysts, classification of, 1
Bromelin, 94
Butylbenzoate synthesis by esterase, 42, 43

C

Cannizzaro reaction, 174
Carbohydrases, 125–147
Carbonic anhydrase, 223–226
  chemical nature, 226
  estimation, 224
  preparation, 223
  specificity, 226
Carrier theory, 19
Catalase, 157
  alleged reversible hydrolysis, 162
  chemical nature, 159, 160
  digestion by trypsin, 161
  formation of enzyme-substrate compound, 162–164
  inhibitors, 161
  kinetics, 158, 159, 162–164
  optimum *p*H, 159
  preparation, 157
Cellulase, 147
Chemistry of milk clotting, 58
Chlorophyllase, 35
Cholesterol-ester synthesis by esterase, 44
Choline esterase, 39
Chymoinhibitors, 56, 57

Chymotrypsin, 72, 73
  activation, 73
Citric dehydrogenase, 172
Coenzymes, 12, 170, 213
Cozymase, 214
  chemical nature, 214
  function, 213
Crystalline carboxypolypeptidase, 92
Crystalline chymotrypsin, 71
Crystalline chymotrypsinogen, 71
Crystalline pancreatic amylase, 144
Crystalline pepsin, 60
Crystalline pepsinogen, 54
Crystalline trypsin, 69
Crystalline trypsinogen, 76
Crystalline urease, 109
Crystalline yellow oxidation enzyme, 205
Cytochrome, 193

D

Dehydrogenases, 168–178
Desmo-trypsin, 68
Dopa oxidase, 185

E

Enterokinase, 67, 68
Enzymes, activators and inhibitors, *see* Activation, Inactivation
  antiseptics, 13
  chemical nature, 17
  combination with substrate, 10
  concentration effect on reaction velocity, 9
  definition, 1
  digestion by proteases, 20
  effect of *p*H, 4
  effect of temperature, 2
  electrolytic nature, 9
  general methods for preparation, 23
  inactivation by proteases, 21
  mechanism of enzyme action, 21
  reversible inactivation, *see* Inactivation
  rule for expressing activity, 12
  specificity, 6
  synthetic action, 22, 23, 42, 43, 170
Enzyme-substrate compound, 10, 162
Emulsin, 134–136
  effect of neutral salts, 136
  specificity, 134–136
Ester synthesis, 42, 43

F

Fermentation, alcoholic, *see* Zymase complex

G

Gastric lipase, 32
Glucose dehydrogenase, 175
Glycerophosphoric dehydrogenase, 173

H

Hexosediphosphoric dehydrogenase, 173
Hexosidases, 125
Hippuricase, 114
Hydrogen-ion dependency, 4

I

Inactivation of enzymes (reversible and irreversible), 2, 12–15, 20, 81, 83, 111, 112, 116, 141, 161, 162, 169, 172, 176, 178, 180, 183, 188, 190, 206, 228
Indophenol oxidase, 187
Inulase, 147
Invertase, 125

K

Kathepsin, 84–88
  activation, by ascorbic acid, 85
    by sulfhydryl compounds, 84, 85
    by HCN, 84, 85
    by natural activator, 85
Keratinase, 94
Kinetics of enzymes, 1–17, 33, 34, 44, 64, 70, 80, 113, 130, 132, 133, 142, 145, 158, 159, 162–164

L

Laccase, 186
Lactase, 131–134
  estimation, 134
  kinetics, 132, 133
  optimum *p*H, 132
  synthetic action, 131

- Lactic acid fermentation, *see* Zymase complex
- Lactic dehydrogenase, 170
- Lichenase, 146
- Liver esterase, 33
  - differences between liver and pancreatic enzyme, 33–34
  - general method for synthesis of esters by esterase, 42
  - induction time, 33
  - kinetics of inhibition, 13
  - synthesis of butylbenzoate, 42
  - synthesis of cholesterol ester, 44
  - temperature coefficient, 4
- Luciferase, 227–229
  - chemical nature, 228
  - mechanism of action, 228
  - preparation, 227
- Luciferin, 228
- Lyotrypsin, 68

M

- Malic dehydrogenase, 170
- Maltase, 129, 130
  - kinetics, 130
  - optimum pH, 130
  - separation from sucrase, 130
  - specificity, 128
  - synthetic action, 130
- Malt amylase, 137–146
  - course of hydrolysis of starch, 145
  - optimum pH, 4, 5, 145
  - separation of alpha and beta amylase, 144
  - temperature coefficient, 2
- Melibiase, 134
- Methylene blue technic for oxidase estimation, 176–178
- Milk-clotting theory, 58
- Monophenol oxidase, 184

O

- Oxidizing enzymes, 167–194
  - theories, 167, 168

P

- Papain, 84–88
  - activation, by blood in cancer, 87
    - by HCN, 84, 85
- Papain, activation, by H_2S, 84, 85
    - by natural activator, 85
    - by sulfhydryl compounds, 84, 85
    - by ultra-violet irradiation, 85
  - labile SH group in enzyme molecule, 84, 85
  - specificity, 86, 87
- Pancreatic amylase, 137, 138–1411, 43
  - optimum pH, 141
  - purification and chemical nature, 143
- Pancreatic enzymes, separation, 66
- Pancreatic "erepsin," 88–95
- Pancreatic lipase, 29
  - optimum pH, 32
  - specificity, 30
  - temperature coefficient, 4
- Pancreatic prolipase, 30
  - activation, by inorganic compounds, 31
    - by various sera, 31
- Pepsin, 53–64
  - acetyl derivatives, 63
  - chemical differences between rennin and pepsin, 55, 56
  - chemical nature, 61, 62
  - crystallization, 60
  - optimum pH, 64
  - rennin-pepsin problem, 53
  - specific inhibitors, 56, 57
  - specificity, 55–58
- Pepsinogen, 53
  - preparation of crystalline pepsinogen, 54
- Peptidases, 88–94
  - aminopolypeptidase, 88
  - carboxypolypeptidase, 92
  - dehydropeptidase, 94
  - dipeptidase, 93
  - pancreatic polypeptidase, 90
  - preparation of crystalline carboxypolypeptidase, 92
  - prolinase, 91
- Peroxidase, 178–184
  - chemical nature, 183
  - color reactions, 179
  - effect of pH, 183
  - function, 178, 179

Peroxidase, interaction with ascorbic acid, 180–182
Phosphatases, 37–38
  in diseases, 38–41
  optimum pH, 37, 38
  phosphodiesterase, 38
  phosphomonoesterase, 37
Polyases, 137–146
Polyphenol oxidase, 186
Protein structure, 49–53
Proteolytic enzymes, 53–95
  classification, 95
  inactivation, 13, 63
  mechanism of action, 9, 10, 49–53, 63
  milk-clotting power, 58, 59
  synthetic action, 22
Purine amidases, 118, 119

R

Rennin, 53, 55, 58, 59
  chemical nature, 55
  digestion, by pepsin, 20
    by trypsin, 20
  estimation, 58
  rennin-pepsin problem, 53, 54
Ricinus lipase, 35

S

Saccharase, 125–129
  see also Sucrase
Salivary amylase, 138, 140
Schardinger enzyme, 174
Schütz' law, 9, 10
Starch hydrolysis, 137–144
Stereospecificity, 6, 30
Succinic dehydrogenase, 169
Sucrase, 125–129
  estimation, 127
  kinetics, 10–12
  optimum pH, 127
  preparation, 125
  specificity, 128
Sulfatase, 36

T

Tannase, 36
Trypsin, 66–84
  action, on ascorbic acid oxidase, 21
    on catalase, 161
    on lipase, 21
    on luciferase, 228
    on maltase, 21
    on pepsin, 21
    on salivary amylase, 21
    on sucrase, 21
    on urease, 21, 111
  activation by enterokinase, 67
  autolytic activation, 67
  autolytic trypsin, 81, 82
  crystalline chymotrypsin, 71, 78
  crystalline trypsin, 69
  desmotrypsin, 68
  difference between trypsin and chymotrypsin, 80–83
  inhibitor, 81
  inhibitor-trypsin compound, 81
  kinetics, 70, 71, 80
  lyotrypsin, 68
Trypsinogen, 76
Tyraminase, 107
Tyrosinase, 184

U

Urease, 107–114
  activators, 111, 112
  adsorption, 109
  chemical nature, 109, 111
  crystallization, 109
  effect, of buffers, 4, 114
    of dyes, 112
    of heavy metal salts, 109
    of pepsin, 111
    of trypsin, 111
    of ultra-violet irradiation, 110
  kinetics, 113
  labile SH groups in enzyme, 112
  mechanism of action, 108
  optimum pH, 4, 114
  physiological properties, 113
  specificity, 109
Uricase, 187

V

Vitamin-A-destroying enzyme, 190
Vitamin B_2 part of yellow oxidation enzyme, 207
Vitamin C oxidase, 188

X

Xanthine dehydrogenase, 174

Y

Yellow oxidation enzyme, 203–209
  components, 204, 205
  crystallization, 205
  formation from synthetic prosthetic group and specific protein, 206
  reversible hydrolysis, 206–208
  vitamin B_2, part of, 206, 207

Z

Zymase complex, 211–219
  components, 211, 212
  cozymase, 214
  induction, 213
  intermediary products of fermentation, 216–219
  rôle of inorganic phosphate, 215
  scheme of Emden, 216
  scheme of Meyerhof, 218

This book must be returned within 3/7/14 days of its issue. A fine of ONE ANNA per day will be charged if the book is overdue.